



# Cellular and Molecular Mechanisms of Chronic Inflammation in Aging of Skeletal Muscle

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**Cellular and Molecular Mechanisms of Chronic Inflammation in  
Aging of Skeletal Muscle**

A dissertation presented

by

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to

**The Division of Medical Sciences**

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Human Biology and Translational Medicine

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## **Cellular and Molecular Mechanisms of Chronic Inflammation in Aging of Skeletal Muscle**

### **Abstract**

Aging of skeletal muscle is typically accompanied by declining regenerative potential, due in part to alternations in the resident muscle stem cell population, known as satellite cells. Previous data suggest that highly regenerative satellite cells in young mice are damaged by aging and chronic inflammation, driven in part by the transcription factor NF- $\kappa$ B. Interestingly, myogenic function of aged satellite cells can be restored by exposure to blood-borne factors from young mice, in association with decreased expression of many of pro-inflammatory genes.

These observations led me to hypothesize that strategies counteracting the chronic inflammatory state in muscle might improve regenerative function in old age through enhancement of satellite cell function. To test this hypothesis, this study aims to define the molecular factors that promote chronic muscle inflammation with aging, evaluate their impact on satellite cell functions, and determine whether inhibition of the inflammatory process indeed reverses age-related muscle dysfunction. Utilization of well-established mouse models reveals that NF- $\kappa$ B activity has a detrimental effect on satellite cell function via non-cell-autonomous mechanisms, and that inhibition of NF- $\kappa$ B activity and its downstream target phospholipase A2 in skeletal muscle fibers preserves muscle regenerative potential in aged animals. In addition, this study reports the restrictive role of IL-6, a pro-inflammatory cytokine widely recognized as a biomarker of chronic inflammation, on the myogenic function of satellite cells. Finally, this study



shows that systemic inhibition of inflammation using the NF- $\kappa$ B antagonist sodium salicylate decreases inflammatory gene expression, including IL-6, in aged muscle and improves muscle regeneration after injury.

Thus, chronic inflammation in muscle, in association with elevated NF- $\kappa$ B activity and its downstream pro-inflammatory factors, impairs muscle regeneration by extrinsically limiting the myogenic function of satellite cells in aged animals. Importantly, such impairment was shown to be reversible by reducing the inflammatory tone both at tissue and systemic level. By discovering the molecular mediators NF- $\kappa$ B signaling in muscle, this study provides potential therapeutic avenues for elderly patients with declining muscle mass and function.

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## List of abbreviations

<b>ATG7</b>	Autophagy related protein 7
<b>CCL2</b>	Chemokine ligand 2
<b>CMA</b>	Chaperone-mediated autophagy
<b>COX</b>	Cyclooxygenase
<b>CRP</b>	C-reactive protein
<b>DDR</b>	DNA damage response
<b>ELISA</b>	Enzyme linked immuno assay
<b>EMSA</b>	Electromobility shift assay
<b>ENCODE</b>	the Encyclopedia of DNA Elements
<b>FACS</b>	Fluorescent activated cell sorting
<b>FDA</b>	U.S. Food and Drug Administration
<b>FSC</b>	Forward scatter
<b>GDF11</b>	Growth differentiation factor 11
<b>GFP</b>	Green fluorescent protein
<b>H&amp;E</b>	Hematoxylin and eosin
<b>HSC</b>	Hematopoietic stem cell
<b>HSP</b>	Heat shock protein
<b>HSP72-tg</b>	Heat shock protein 72-overexpression transgenic mouse
<b>Ikb</b>	Inhibitor of kB
<b>IL-6</b>	Interleukin 6
<b>IL-6 KO</b>	Interleukin-6 knockout mouse
<b>IRES</b>	Internal ribosome entry site
<b>JAK</b>	Janus kinase

<b>MCP-1</b>	Monocyte chemotactic protein 1
<b>MISR</b>	Muscle specific I $\kappa$ B super repressor
<b>MuRF-1</b>	Muscle ring finger protein-1
<b>NF-<math>\kappa</math>B</b>	Nuclear factor $\kappa$ B
<b>PLA2</b>	Phospholipase A2
<b>PLA2G5</b>	Phospholipase A2 group 5
<b>PG</b>	Prostaglandin
<b>SASP</b>	Senescence associated secretory phenotype
<b>SC</b>	Satellite cell
<b>SC-IKK</b>	Satellite cell-specific IKK $\beta$ activation mouse
<b>SEM</b>	Standard error of mean
<b>SSC</b>	Side scatter
<b>siRNA</b>	Short interference RNA
<b>STAT3</b>	Signal transducer and activator of transcription 3
<b>TA</b>	Tibialis anterior
<b>TNF<math>\alpha</math></b>	Tumor necrosis factor alpha
<b>WT</b>	Wildtype

**Chapter 1.**  
**Introduction**

## ***Population Aging***

Aging is a universal, natural biological process attributed to cumulative changes in molecular and cellular structures that disrupt organismal homeostasis over the passage of time. As a result, aging animals experience progressive decline in tissue function and impaired regeneration, which cause functional limitations and increased vulnerability for chronic diseases, disability and mortality (Seals, 2015). Medical advances and socioeconomic development in the 20<sup>th</sup> century have greatly improved global health status and increased life expectancy. In the past 100 years, global life expectancy has nearly doubled, from 34 to 67 (Riley, 2013). As this trend continues, the composition of the world's population is expected to show a proportionate shift from younger to older people. The worldwide numbers of those aged 60 and above will rise to 21% of population by 2050, comprising the same percentage of the population as those under age 15 (Harper, 2014). The proportion of older adults will be even higher in developed countries; at least 25% of the population will be older than 65 years of age by the year 2050, with some regions exceeding 40% (Lutz, 2008; Petsko, 2008).

Since aging itself is a risk factor for a myriad of diseases that range from neurodegenerative disorders to osteoporosis and cancer, rapid aging of the world's population presents certain socio-economic challenges, such as greater caregiving and financial burden for families, demand for residential housing and pressure on government health insurance programs (Olshansky, 2009; Beard, 2014; Harper, 2014). Moreover, the speed of population aging is projected to accelerate in the next century with further increases in life span and decreases in fertility. As of 2010, the United States houses over forty million people above the age of 65, and this number is expected to more than double by 2050, with the greatest rate of increase in the oldest population aged 80 or over (Vincent, 2010). Current demographic trends in the U.S indicate that the U.S. Social Security Administration may be underestimating the

increase in life expectancy between the present and 2050 by up to 7.9 years, resulting in a possible unanticipated \$1.7 trillion to \$4.4 trillion increase in Social Security costs (Olshansky, 2009).

In light of the considerable burden population aging places on society, it is no wonder that research into aging and its effects has virtually exploded in the past thirty years. In 1980, James Fries proposed “compression of morbidity” as a promising strategy to address the problem. His proposal suggests that the cost of extended life span could be reduced by redirecting the focus of health improvement to postponing the age of onset of chronic diseases rather than surviving longer, in order to minimize the amount of disability and the span of morbidity before the natural occurrence of death (Fries, 1980). More recently, leading scientists in the field of biological aging research have adopted the idea of compression of morbidity and promoted the concept of healthy aging, which aims for the extension of “healthspan.” (Kirkland, 2009; Seals, 2014). The general goal is to live long, but with good health and high quality of life.

Age-associated physiological dysfunctions are a major obstacle for maintaining quality of life into old age. In particular, loss of muscle strength can affect overall quality of life by limiting motility and the ability to perform basic activities of daily living, often leading to more adverse consequences such as falls, fractures, even mortality (Hanna, 2015). In fact, loss of muscle mass and strength is thought to be a major contributor to the development of frailty, a common clinical syndrome in older adults that carries an increased risk for poor health outcomes (Xue, 2011; Clegg, 2011).

Based on these observations, I seek to focus specifically on the effect of systemic aging on skeletal muscle in this research, by probing the molecular mechanism(s) that may serve as a fundamental cause of age-related decline in skeletal muscle function. This study aims for a better understanding of muscle aging, which will ultimately contribute to the development of therapeutic strategies for healthy aging.

### ***Sarcopenia: Aging of Skeletal Muscle***

One of the major effects of aging is a decline in tissue regeneration after damage. This is especially true in the case of skeletal muscle, which loses the ability to repair itself later in life (Grounds, 1998; Wagers, 2005). Skeletal muscle, a self-repairing tissue that allows for precise movement, coordination, and force production, makes up over 30% of the human body by weight (Janssen, 2000). The decrease in regenerative potential of skeletal muscle and reduction in muscle tissue quality inevitably results in sarcopenia, an age-dependent loss of muscle mass and function. Sarcopenia is characterized by progressive muscle atrophy, with an estimated 0.5%-1.5% loss of muscle mass each year starting from the age of 50, an increased fat:muscle ratio, and changes in muscle architecture and neuromuscular junctions (Ryall, 2008). Sarcopenia exhibits itself in debilitating traits such as a stooped posture and weakness that increase risk for incident disability, all-cause mortality, mobility disability, and loss of independence, affecting about 40% of people over the age of 80. Furthermore, sarcopenia is frequently observed in association with other age-related chronic diseases such as type II diabetes, osteoporosis, cancer and obesity, exacerbating these pathological states (Dutta, 1997; Kim, 2009). Prevalence of sarcopenia continues to increase as the global population ages; however, currently there are no treatment options for this disease (Baumgarner, 1998).

Multiple, interrelated factors contribute to the development and progression of sarcopenia. Physical inactivity due to illness or hospitalization, altered hormonal status (decreased levels of testosterone, androgen, growth hormones), insufficient dietary nutrients due to decreased food intake and dysregulation of appetite, changes in the circulating levels of catabolic inflammatory mediators, and inefficient protein synthesis have been postulated as major factors involved (Doherty, 2003; Fielding, 2011). Also, age-related loss of motor units, changes in structural and functional integrity of the neuromuscular junction and functional

denervation have been well documented to promote muscle weakness and fatigue, preceding muscle wasting associated with sarcopenia (Belluardo, 2001; Jang, 2011; Gutmann, 1973). At the cellular level, sarcopenia manifests with a reduction in the size of each individual myofiber and of the total number of myofibers, multi-nucleated cells that serve as individual components of skeletal muscle (Faulkner, 2007). Decreased myofiber size in aged skeletal muscle is followed by a loss of myonuclei per unit length of myofiber (Brack, 2005), and decreased number of myonuclei led to a hypothesis that sarcopenia may be caused by loss of muscle stem cells, or commonly referred to as satellite cells. In fact, cross-sectional analyses on the characteristics of skeletal muscle in elderly men showed positive correlations among the cross-sectional area of type II myofibers (fast-twitch muscle fibers), myonuclear content, and satellite cell content, providing further support to the idea that satellite cell content plays an important role in regulating myofiber size, muscle mass and strength in older men (Verdijk, 2010). Also a recent study revealed severely impaired regeneration and increased muscle fibrosis in sedentary adult mice with inducible, life-long reduction of satellite cell population, proving the requirement of satellite cell in muscle regeneration (Fry, 2015).

### ***Age-dependent decline in stem cell functionality of satellite cells***

Satellite cells are known to enable muscle regeneration by proliferating and differentiating into myoblasts in response to muscle injury, but are otherwise mitotically quiescent (Montarras, 2005; Sambasivan, 2011). Myoblasts differentiated from satellite cells during muscle regeneration are incorporated into existing myofibers or fuse with others to form new myofibers (Schultz, 1985; Bischoff, 1994) (Figure 1). Also, the residual pool of quiescent satellite cells is maintained for additional rounds of regeneration by symmetric and asymmetric self-renewal of satellite cells (Moss, 1971; Kuang, 2007). The presence of satellite cells was first discovered by Alexander Mauro in 1961, who noticed its unique placement on the sarcolemma



and under the basal lamina, two thin membranes that surround myofibers (Mauro, 1961). Satellite cells have since been characterized as unipotent adult muscle stem cells.

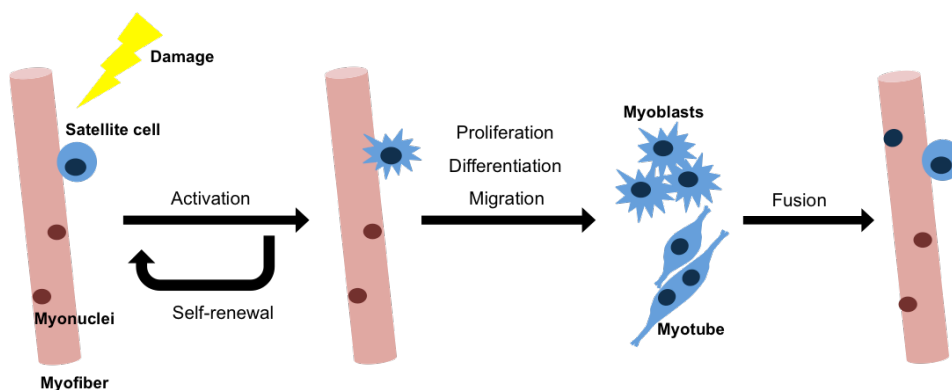


Figure 1. Skeletal muscle regeneration after damage

Satellite cells are characterized by expression of the paired-box transcription factor Pax7, Pax3, and myogenic regulatory factor Myf5 (Buckingham, 2007; Seale, 2000; Cornelison, 1997). Although multiple studies have reported heterogeneous expression of Pax3 and Myf5 in satellite cells, Pax7 is considered to be the canonical biomarker of satellite cells as it is expressed in quiescent and activated, proliferating satellite cells across multiple species (Sacco, 2008; Yin, 2013). Pax7 also functions as a regulator of satellite cell survival, proliferation, and prevents precocious differentiation of myogenic progenitor cells (Oustanina, 2004; Relaix, 2006; Zammit, 2006).

Several groups including the Wagers lab have reported age-associated reduction in the number of satellite cells and hypothesized that this loss of satellite cells ultimately contributes to the aged muscle phenotype (Collins, 2007; Shefer, 2010; Sinha, 2014). Other studies have demonstrated that effective muscle regeneration hinges on the presence of satellite cells, making this hypothesis highly plausible (Sambasivan, 2011; Lepper, 2011). In addition, the

Wagers Lab and several other groups have found that the satellite cells still remaining in aged muscle fail to respond adequately to muscle injury and have reduced myogenic potential (Sinha, 2014; Sousa-Victor, 2014; Cosgrove, 2014). For better understanding of the mechanisms underlying this age-related process of muscle loss, active investigation into the phenomena of sarcopenia has been conducted in the field.

Aging of satellite cells can be attributed to age-related changes at many levels. It has been suggested that the decline in stem-cell functionality of satellite cells with age may be caused by intrinsic molecular changes in aged muscle stem cells or extrinsic changes due to the aging of the local microenvironment (e.g. myofibers and the extracellular matrix) or the systemic environment (e.g. changing levels of cytokines in the circulatory system) (Rando, 2006). Since satellite cells exist predominantly in a quiescent state, it is likely that they exhibit a weaker stress response and lower levels of DNA damage repair proteins that are characteristic of non-dividing cells, thereby accumulating excessive oxidative damage and protein aggregates over time (Bohr, 2002). The intracellular amassment of damage can then lead to cellular senescence or apoptosis, reducing the pool of available satellite cells and thus inhibiting effective muscle regeneration (Sousa-Victor, 2014).

Multiple groups have made use of the parabiotic model, in which the circulatory system of two animals are connected, to show that aged progenitor cells can be rejuvenated extrinsically, by exposure to a young systemic environment (Conboy, 2005; Sinha, 2014). In particular, decreased Notch signaling and the ensuing excess expression of the circulating growth factor TGF- $\beta$  have been implicated in the failure of muscle stem cells to activate in response to injury (Carlson, 2009). Carlson et al. demonstrated that this reduction in regenerative potential can be reversed by activating MAPK/Notch in an *in vitro* study using cultured human muscle stem cells (Carlson, 2009). These studies suggest that age-associated loss of regenerative potential in skeletal muscle results from loss or dysfunction of muscle stem

cells and that restoration of proper stem cell activity would rejuvenate regenerative function in aged muscle.

### ***Inflamm-aging: Chronic Inflammation in the Elderly***

An important systemic change that is thought to have a profound role in the aging process is chronic, low-grade inflammation, characterized by persistently elevated serum levels of pro-inflammatory cytokines even without overt infection. Inflammation is beneficial as an acute, transient response to injury or pathogens, but chronic inflammation has been found in close relation to many age-related diseases, including Parkinson's, Alzheimer's, cancer, diabetes, arthritis and sarcopenia (Di Iorio, 2006; Sarkar, 2006; McGeer, 2003; McGeer, 2004). The detrimental effects of chronic inflammation are mediated by the immune system in a process has been termed, "inflamm-aging" (Franceschi, 2000). Franceschi et al (2007) argue that inflamm-aging is evidence for a theory of aging called antagonistic pleiotropy, in which the expression of genes favorable at a young age are no longer beneficial at an old age, resulting in a decline in tissue function. Antagonistic pleiotropy calls for an evolutionary perspective on aging. From this point of view, the human immune system was evolutionarily selected and perfected to combat acute infections in youth in order that a child might live long enough to reproduce. However, with the advancement of agricultural, industrial, and medical technologies, *Homo sapiens* are living longer and longer, with immune systems unfit to deal with chronic diseases. The aging process is simultaneously accompanied by characteristics that accelerate the pro-inflammatory signals and counteracting anti-inflammatory characteristics. The balance between these opposite features controls the outcome of the aging process, either leading to frailty and degenerative diseases or a healthy old age and longevity. In the process of inflamm-aging, adaptive immunity declines, and the anti-inflammatory mechanisms are thought to be insufficient and ill-equipped to deter inappropriately activated innate immune responses,

resulting in mild hyper-activity of circulating inflammatory factors. However, the precise etiology of inflamm-aging and its potential causal role in contributing to adverse health outcomes are still under investigation.

In addition to age-dependent changes in the immune system, there are a number of potential drivers of inflamm-aging, which are also closely related to one another (Franceschi, 2014). Accumulation of damaged macromolecules and cellular components constantly causing the initiation of immune response has been proposed as a potent contributor of chronic inflammation. Reactive molecules generated in aged cells over time as a byproduct of normal metabolism or from extrinsic paracrine and endocrine mediators are thought to drive a vicious cycle that causes damages to nucleic acids, proteins, and lipids, and disrupts mitochondrial oxidative phosphorylation, producing additional metabolic byproducts (Harman, 1975). As a response to the cellular damages and stress, aged cells readily undergo senescence, which is also considered to drive aging and age-associated pathologies through its secretory phenotype known as senescence-associated secretory phenotype (SASP) (Campisi, 2007; Baker, 2011). As SASP includes numerous pro-inflammatory cytokines, accumulation of senescent cells in aged animals could trigger chronic inflammation.

These stimulators of chronic inflammation converge on few molecular pathways such as activation of NLR family-pyrin domain containing 3 (Nlrp3) inflammasome and nuclear factor  $\kappa$ B (NF- $\kappa$ B) (Iyer, 2013; Bollrath, 2009). In particular, activation of the NF- $\kappa$ B pathway has been known as a transcriptional signature of chronic inflammation in aged skin, skeletal muscle, bone, and nervous system (de Magalhães, 2009), although the direct effects of NF- $\kappa$ B activation on the stem cell function in these tissues is still under investigation. Considered a common mediator of the various inputs and responses to cellular damage, NF- $\kappa$ B is activated by most inflammatory agents. Salminen et al. (2008) claim that NF- $\kappa$ B is the culprit of “inflamm-aging” through its effects on circulating levels of pro-inflammatory cytokines, such as interleukin 6 (IL-

6), a common biomarker of inflammatory status and a hallmark of chronic morbidity. IL-6 is also associated with and predictive of many aging phenotypes, for example, changes in body composition, energy production and utilization, metabolic homeostasis, immune senescence, and neuronal health (Franceschi, 2014).

### ***Reversing Aging by Restoring Stem Cell Function***

Strong evidence that aging of stem cells involves dominant signals from the local and systemic environment comes from studies in mice in which surgical and genetic systems have been used to modulate the aged tissue and systemic milieu. In particular, exposure of old skeletal muscle to a youthful systemic environment through heterochronic parabiosis can promote efficient satellite cell activation in old muscle (Conboy, 2005). This surgical intervention also enhances rates of neurogenesis, remyelination and other measures of neural function in the old parabiont, suggesting that changes in stem cell function in the aging central nervous system are also affected by blood-borne factors (Villeda, 2011; Katsimpardi, 2014; Ruckh, 2012). Intriguingly, at least part of the ‘rejuvenating’ effect of a young systemic environment appears to relate to the higher levels of circulating growth differentiation factor 11 (GDF11) and oxytocin present in young blood as compared to aged (Sinha, 2014; Katsimpardi, 2014; Loffredo, 2013; Elabd, 2014). Treatment of aged mice with either recombinant GDF11 or oxytocin reverses dysfunction of aged satellite cells and restores robust regenerative function in aged mice (Sinha, 2014; Elabd, 2014). GDF11 supplementation in old mice further reverses age-related hypertrophy in cardiac muscle (Loffredo, 2013), enhances neural stem cell and neuronal function (Katsimpardi, 2014) and improves physical activity (Sinha, 2014). These studies raise the possibility that manipulation of blood-borne factors could provide a particularly attractive strategy for the treatment of age-related muscle disease.

In addition to systemic factors, improving protein homeostasis could also help restore stem cell function. Up-regulation of pro-autophagy genes by FOXO3A was shown to be essential for protecting aged HSCs from metabolic stress (Warr, 2013). Pharmaceutical inhibition of mTOR has been reported to facilitate autophagosome formation (Nazio, 2013; Kim, 2011) and also to restore the self-renewal and hematopoietic potential of aged HSCs (Chen, 2009). Likewise, data from mice genetically manipulated to preserve chaperone-mediated autophagy (CMA), a form of autophagy involving chaperone-dependent selection of substrates, showed improved cellular homeostasis, enhanced resistance to stressors and preservation of organ functions with age (Zhang, 2008). Overexpression of heat shock protein 70 (HSP70), a chaperone protein that recognizes damaged proteins in CMA, also protects against age- and disease-related tissue degeneration in brain, heart and skeletal muscle (Cummings, 2001; Feng, 2014; McArdle, 2004). These studies provide intriguing insights into potential therapeutic interventions for age-related dysfunction, although the specific role, if any, of CMA and chaperone proteins in stem cell aging is a subject for future research.

In this study, I hypothesize that chronic inflammation is a main contributor to satellite cell dysfunction in aging, and aim to understand the underlying mechanism by defining molecular factors involved in age-associated chronic inflammation in skeletal muscle. In particular, the NF- $\kappa$ B pathway is assessed as a major mediator of chronic inflammation. Utilizing genetic and pharmacological modulation of NF- $\kappa$ B activity in muscle tissue or in the whole body of mice, this study demonstrates the impact of NF- $\kappa$ B-mediated chronic inflammation on the functionality of muscle stem cell and its niche. Furthermore, this study examines the reversal of chronic inflammation as a strategy to restore myogenic regenerative potential and limit age-related muscle loss.

## **Chapter 2.**

**NF- $\kappa$ B mediates chronic inflammation in aged muscle and  
impairs muscle stem cell niche**

## ***Preface***

In skeletal muscle, age-associated loss of regenerative potential results in delayed or deficient myogenesis, replacing damaged muscle fibers with fat and fibrous tissue rather than newly formed muscle (Conboy, 2005a) and slowing recovery of muscle function (Blau, 2014). The mechanisms underlying age-related muscle dysfunction remain incompletely understood, but some studies have implicated loss or functional impairment of satellite cells within aged muscle (Sherwood 2004; Conboy 2003). Notably, age-related decline in satellite cell myogenic potential appears to be reversible, as demonstrated by improved muscle regeneration following heterochronic parabiosis, or systemic administration of growth differentiation factor-11 or oxytocin (Conboy, 2005b; Sinha, 2014; Conboy, 2014).

One possible cause for deficient satellite cell function in aged muscle is an increase in skeletal muscle inflammation, which may be mediated in part by increased transcriptional activity of NF- $\kappa$ B (Barnes, 1997; Cai, 2004; He, 2013). Studies of aged and sarcopenic humans support the association of increased “inflammatory tone” in muscle with reduced physiological function (Barnes, 1997). Studies in mice also support the notion that a pro-inflammatory microenvironment may limit repair potential in aged muscle (Tierney, 2013, Oh, 2014).

NF- $\kappa$ B is a transcription factor that plays a central role in modulating multiple systemic and cellular functions, including inflammation, immunity, cell survival, and proliferation (Karin, 2006). Inactive NF- $\kappa$ B remains in the cytoplasm bound to inhibitor of kappaB (I $\kappa$ B); however, I $\kappa$ B kinase b (IKKb) becomes activated in response to pro-inflammatory factors, including tumor necrosis factor alpha (TNF $\alpha$ ) and IL-6, and subsequently phosphorylates I $\kappa$ B, leading to its ubiquitination and degradation (Hayden, 2004). Degradation of I $\kappa$ B releases NF- $\kappa$ B, which translocates to the nucleus and transcriptionally upregulates pro-inflammatory effector genes. NF- $\kappa$ B activity is transiently regulated under normal physiological conditions, but constitutive



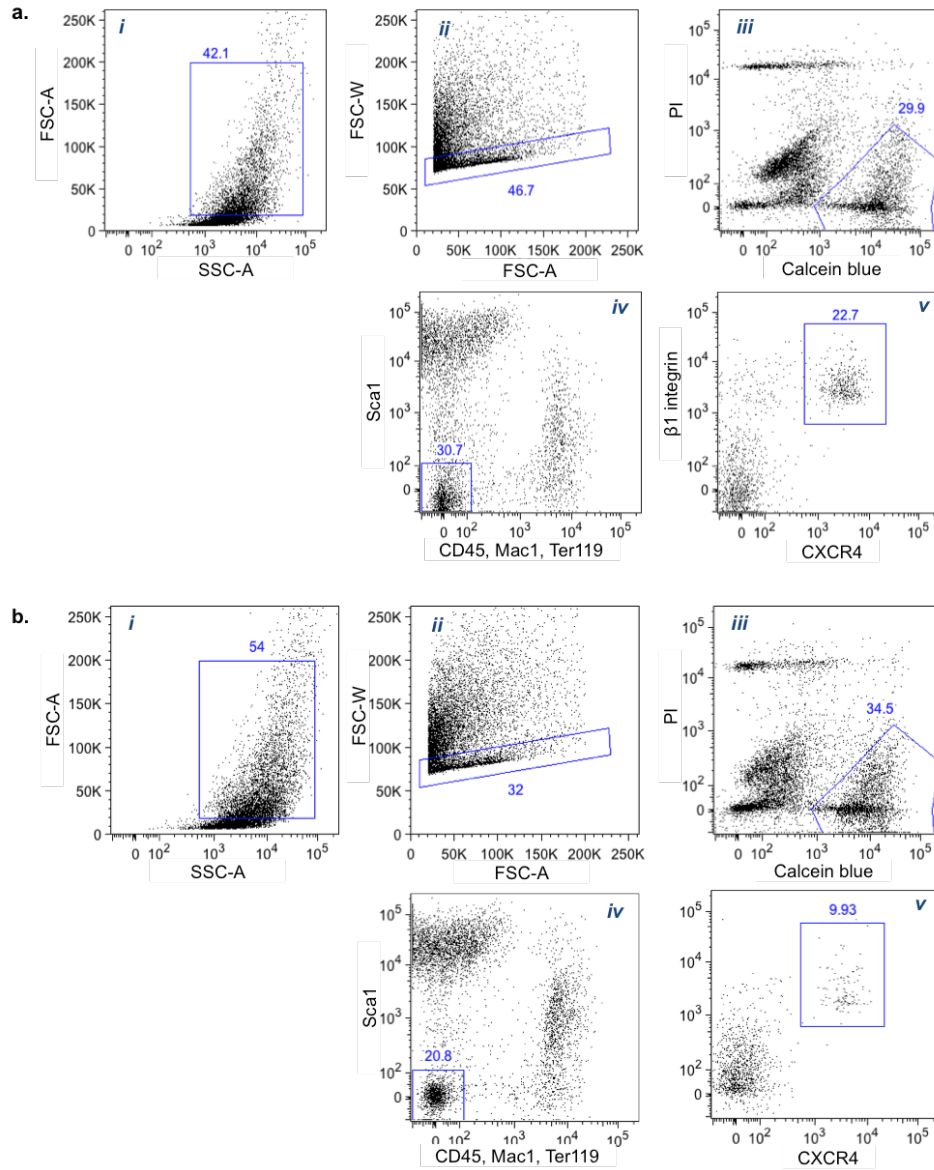
activation of NF- $\kappa$ B has been observed in several disease states, including muscle wasting and muscular dystrophy (Li, 2008; Cai, 2004; Acharyya, 2007).

NF- $\kappa$ B activation has been shown to inhibit *MyoD* expression and thereby repress myofiber formation in culture (Guttridge, 2000; Langen, 2004). Yet, how NF- $\kappa$ B may function to regulate skeletal muscle regeneration *in vivo* is not as well understood. Furthermore, although increased NF- $\kappa$ B activity is associated with sarcopenia (Bar-Shai, 2005; Giresi, 2005; Cai, 2004), its role in aging-associated satellite cell dysfunction has not been clarified. In this chapter, I analyze changes in NF- $\kappa$ B activity that occur with skeletal muscle aging, examine the relevance of NF- $\kappa$ B signaling in skeletal muscle satellite cell function and muscle regeneration, and determine whether pharmacologic inhibition of NF- $\kappa$ B can improve muscle repair activity through stimulation of satellite cell function.

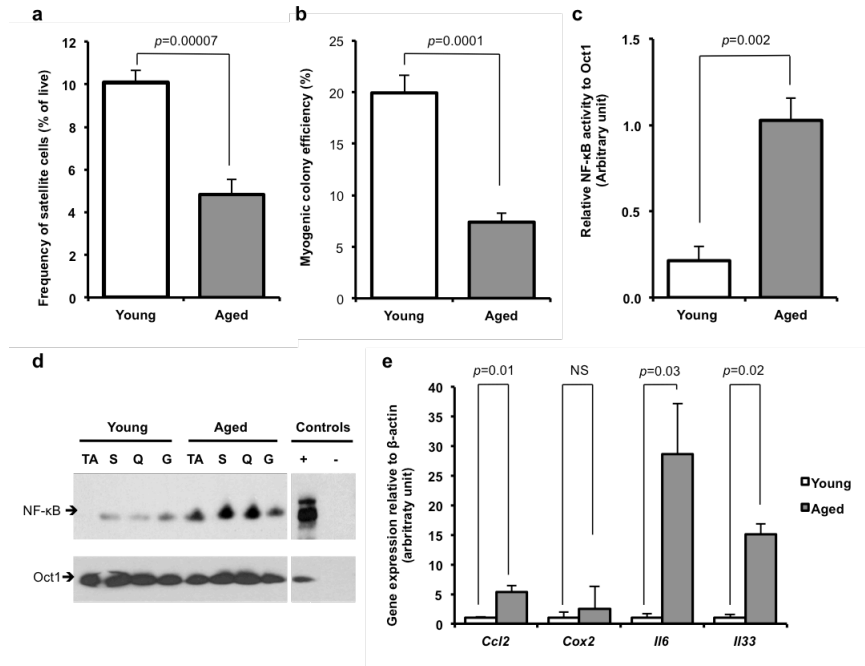
## **Results**

### ***Satellite cell frequency and survival decrease with aging.***

Satellite cells in this study were isolated by fluorescence-activated cell sorting (FACS) as the CD45<sup>-</sup>Sca1<sup>-</sup>Mac1<sup>-</sup>CXCR4<sup>+</sup> $\beta$ 1integrin<sup>+</sup> subset of myofiber associated cells, as previously described (Sherwood, 2004; Cerletti, 2008). Representative FACS schemes for isolation of young and aged satellite cells are shown in Figure 2. For evaluation of stem cell functionality of satellite cells in young and old mice, frequency of satellite cell and myogenic colony formation potential was analyzed. Satellite cell frequency was calculated as percentage of CD45<sup>-</sup>Sca1<sup>-</sup>Mac1<sup>-</sup>CXCR4<sup>+</sup> $\beta$ 1integrin<sup>+</sup> population in myofiber-associated cells that are positive for cell viability marker, calcein blue. For myogenic colony formation assay, single satellite cell was seeded into each well of 96-well culture plates and was allowed to form colony. After isolation and culture, single satellite cell is activated rapidly to expand and transition to the progenitor stage to undergo myogenic lineage progression. At day 5 of culture, number of myogenic colony



**Figure 2.** Frequency of satellite cells within the myofiber-associated cell population in young versus aged mice. Representative flow plots depict the sequential gating strategy (*i, ii, iii, iv, v*) utilized and in young (a) and aged (b) mice. Numbers represent percentage of previously gated population. Old mice demonstrate decreased frequency of CD45-Sca1-Mac1-CXCR4+ $\beta 1$ integrin+ satellite cells. Young mice ranged in age from 8-11 weeks. Aged mice ranged in age from 23-26 months.



**Figure 3.** Decreased frequency and survival of satellite cells in aged mice correlate with upregulation of NF-κB activity in aged skeletal muscle. (a) Flow cytometric analysis demonstrates an ~2-fold decrease in the frequency of satellite cells in aged mice as compared to young mice. (b) Double-sorted satellite cells from aged mice exhibit a 3-fold decrease in myogenic colony-forming efficiency as compared to cells isolated from young mice. (c,d) EMSA reveals increased NF-κB activity in hind limb skeletal muscle of aged as compared to young mice. Data are summarized for analysis of fast and slow-twitch hindlimb muscles. S: soleus (slow-twitch); TA: tibialis anterior, Q: quadriceps, G: gastrocnemius (fast twitch). (e) Quantitative RT-PCR analysis of inflammatory genes related to the NF-κB pathway in satellite cells from young or aged mice (n=4-6 samples per gene). *CCL-2*, *Cox-2*, and *IL-6* are transcriptionally upregulated by NF-κB, whereas *IL-33* and leptin receptor mediate increased NF-κB activation. Young mice ranged in age from 8-11 weeks. Aged mice ranged in age from 22-24 months. All data plotted as mean + SEM.

was counted to assess myogenic colony formation efficiency as a measure of satellite cell's ability to survive and proliferate to form myogenic colony in response to signals that stimulate myogenesis (Cerletti, 2008; Cerletti, 2012; Sinha, 2014). Satellite cell frequency within the myofiber-associated cell pool and myogenic colony forming potential are greatly decreased in aged mice (n=12) as compared to young (n=9, Figure 3a,b). These data are consistent with previously reported studies from our lab (Cerletti, 2012; Sinha, 2014) and others (Conboy, 2005). Electrophoretic mobility shift assays (EMSA) confirmed that these differences in satellite cell activity are associated with an overall increase in NF-κB activity in aged skeletal muscle

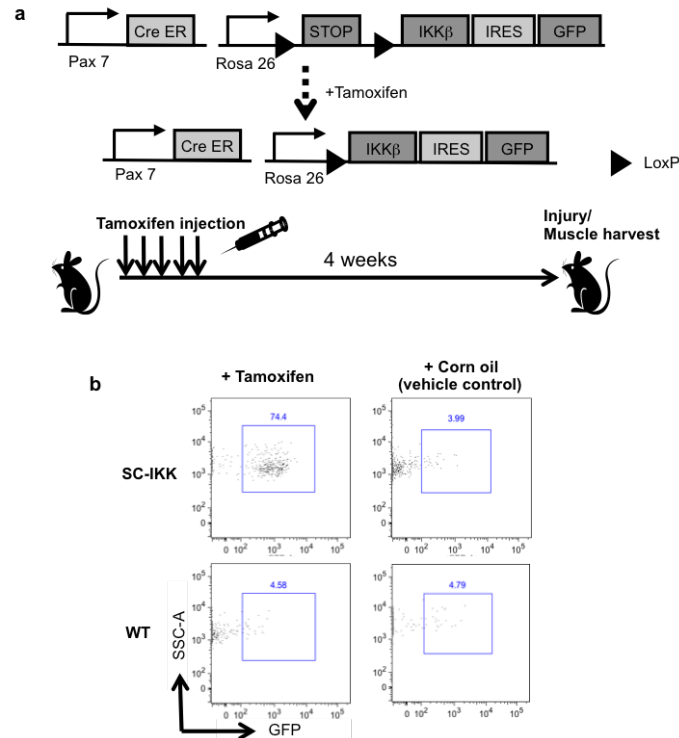
(n=8) compared to young skeletal muscle (n=6, Figure 3c,d). Consistent with enhancement of NF- $\kappa$ B activity in aged satellite cells, many genes that are either direct targets or activators of the NF- $\kappa$ B pathway, including IL-6, IL-33, chemokine-chemokine ligand (Ccl)-2, and cyclooxygenase (Cox)-2 showed transcriptional upregulation in satellite cells isolated from aged as compared to young controls (n=4-6, Figure 3e). Importantly, while prior studies have reported increased NF- $\kappa$ B activity in sarcopenic muscle (Giresi, 2005; Barnes, 1997), this study is the first, to my knowledge, that correlates an upregulation of NF- $\kappa$ B activity with increased expression of pro-inflammatory cytokines and decreased myogenic potential specifically in satellite cells.

***Satellite cell-specific activation of NF- $\kappa$ B activity reveals a non cell-autonomous effect of NF- $\kappa$ B on satellite cell function.***

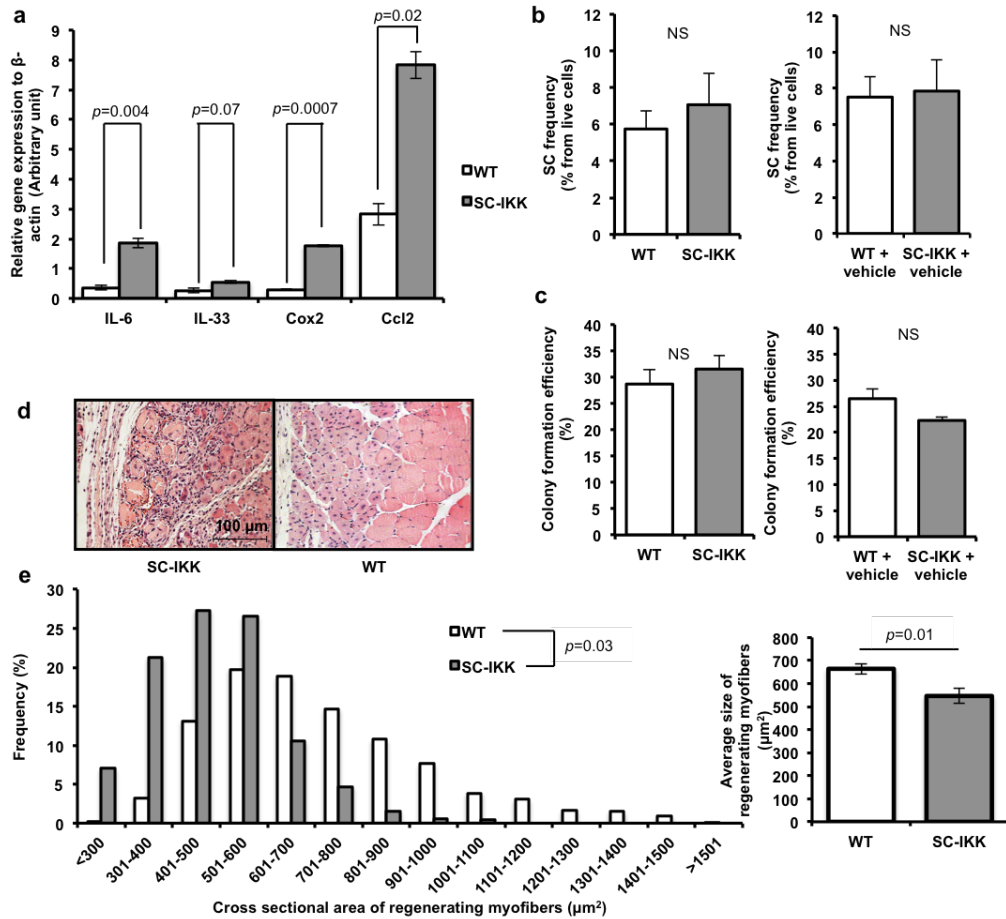
To determine if increased NF- $\kappa$ B activity in satellite cells might directly cause impairment of their regenerative function, I next developed a novel Cre-lox transgenic mouse, engineered to activate NF- $\kappa$ B upon administration of tamoxifen. Experimental animals for this study were generated by breeding mice carrying a tamoxifen-inducible CreER allele driven from the endogenous Pax7 locus (Nishijo, 2009) with mice carrying a loxP-STOP-loxP IKKb and green fluorescent protein (GFP) reporter allele controlled by the ubiquitously expressed Rosa26 promoter and linked by an internal ribosome entry site (IRES) (Figure 4a). Transgenic animals resulting from these crosses enable satellite cell-specific activation of NF- $\kappa$ B signaling by virtue of Cre-dependent expression of a constitutively active IKKb only in Pax7<sup>+</sup> cells, which ensures continuous degradation of I $\kappa$ B and release of NF- $\kappa$ B from its inhibitory complex (Sasaki, 2006). I have called these animals SC-IKK mice for 'satellite cell' specific IKKb production. FACS analysis of myofiber-associated cells harvested from SC-IKK mouse muscle 4 weeks after tamoxifen treatment demonstrated that 61.8% ( $\pm$ 6.4%, n=8) of immunophenotypically identified

satellite cells were GFP<sup>+</sup>, indicating Cre-induced activation of expression of the IKKb-IRES-GFP reporter (Figure 4b). Cell populations of other lineage (fibro-adipogenic, and hematopoietic) were not GFP<sup>+</sup> (data not shown).

To evaluate the impact of constitutive NF- $\kappa$ B signaling in satellite cells on satellite cell phenotype and function, I first examined the frequency and function of these cells in SC-IKK mouse muscle 4 weeks after tamoxifen administration. qRT-PCR analysis demonstrated high levels of inflammatory gene expression in satellite cells harvested from these mice (n=5, Figure 5a). However, in contrast to my hypothesis that activation of NF- $\kappa$ B in satellite cells might cause functional impairment of satellite cells, SC-IKK satellite cells exhibited no change in the overall frequency of satellite cells isolated from young tamoxifen-treated SC-IKK mice (n=8, Figure 5b) and no difference in the ability of these cells to initiate clonal myogenic cell growth in culture (n=8, Figure 5c). Thus, tamoxifen-dependent transgenic induction of NF- $\kappa$ B activity in SC-IKK satellite cells, while sufficient to upregulate NF- $\kappa$ B target genes, does not appear to cause disruptions in satellite cell number or intrinsic myogenic function. On the other hand, when challenged by muscle injury, SC-IKK mice demonstrated a clear deficit in muscle regeneration, evidenced by reduced cross-sectional area of newly formed myofibers (marked by central nuclei, n=7, Figure 5d,e). SC-IKK muscle also showed a decrease in total area of muscle regeneration, with more necrotic fibers and infiltrating inflammatory cells remaining as compared to WT controls at the same time point after injury (Figure 5d, Supplemental figure 1).



**Figure 4.** Satellite cell-specific activation of NF- $\kappa$ B activity. (a) Experimental design. SC-IKK mice contain a floxed-IKK gene under the control of the Pax7 promoter. Exposure of these mice to tamoxifen causes satellite cell-specific expression of IKK $\beta$  and thus a satellite cell-specific increase in NF- $\kappa$ B activity. For these experiments, all mice received vehicle or tamoxifen at 8-9 weeks of age. 4 weeks later, they underwent cryoinjury. SC-IKK and WT mice ranged in age from 14-15 weeks at start of the experiment. (b) Satellite cells from tamoxifen treated SC-IKK mice show transgene-induced GFP expression, which is not seen in vehicle treated SC-IKK controls.



**Figure 5.** Satellite cell-specific activation of NF-κB activity does not affect satellite cell frequency or myogenic colony formation efficiency, but impairs muscle regeneration after injury. (a) Satellite cells from SC-IKK mice demonstrate increased expression of NF-κB target genes following tamoxifen treatment. (b) The frequency of satellite cells and (c) satellite cell myogenic colony formation efficiency are unperturbed in SC-IKK mice or SC-IKK injected with vehicle only. (d,e) Tamoxifen-treated SC-IKK mice exhibit poor skeletal muscle regeneration. Muscle regeneration was assessed by H&E staining at 7 days after cyroinjury and compared to age-matched WT controls. All data are presented as mean  $\pm$  SEM.

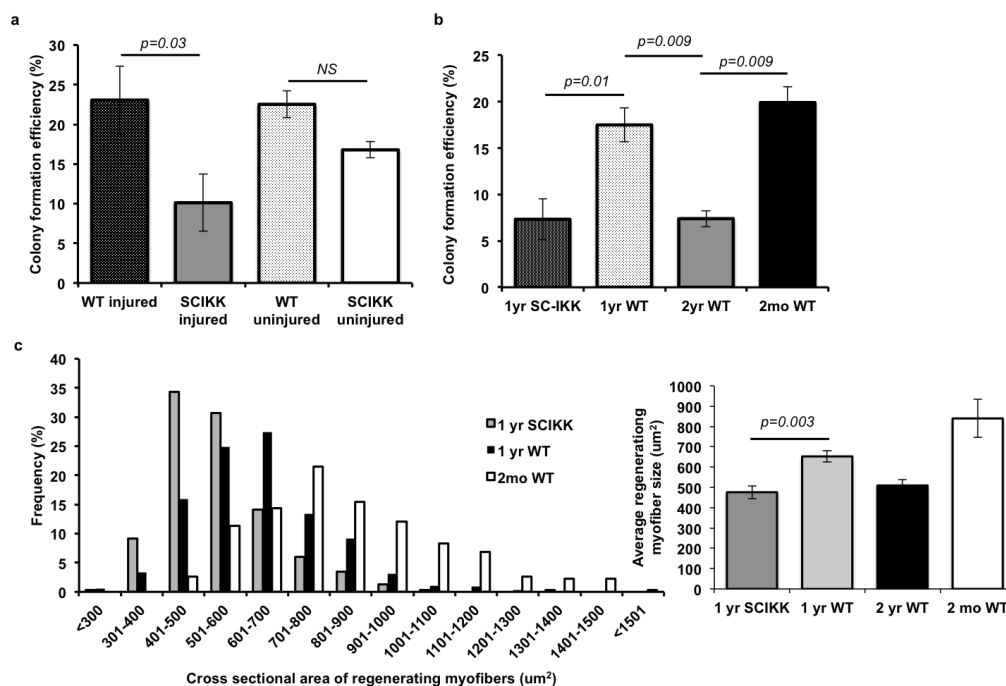
These seemingly contradictory results can be explained if the activated NF- $\kappa$ B exerts its detrimental effect in a non-cell-autonomous fashion. In this model, activation of NF- $\kappa$ B in satellite cells would not affect the cells directly, but when those satellite cells are activated and undergo myogenic differentiation to contribute their nuclei to myofibers, they produce an NF- $\kappa$ B-activated microenvironment that can secrete inflammatory signals and indirectly affect muscle stem cell function, causing impaired muscle regeneration. It is also possible that NF- $\kappa$ B only affects later stages of myogenesis, in which myoblasts fuse to form myotubes and grow in size, either intrinsically or extrinsically. NF- $\kappa$ B-induced loss of MyoD messenger RNA in the C2C12 myoblasts studied by Guttridge et al. (2000) supports the latter explanation, although the specific timing during skeletal muscle development at which NF- $\kappa$ B plays its role is yet to be clarified. In addition, cell culture of SC-IKK satellite cells showed a trend towards decreased differentiation potential compared to WT controls, as assessed by the number of fusion events and size of myotubes, consistent with the notion that constitutive activation of NF- $\kappa$ B in satellite cells can negatively influence the myogenic differentiation of these cells or their progeny (Supplemental figure 2).

***NF- $\kappa$ B-activated niche causes deficits in satellite cell function in young and mid-aged mice.***

To test the aforementioned model of non-cell autonomy of the effect of NF- $\kappa$ B in muscle, I examined the myogenic function of satellite cells isolated from injured muscles of SC-IKK mice. Upon injury, satellite cells of SC-IKK muscle will be activated and differentiate to regenerate myofibers, such that these progeny of SC-IKK satellite cells eventually form a niche with activated NF- $\kappa$ B signaling. Satellite cells from injured SC-IKK muscle indeed showed decreased myogenic efficiency compared to those from injured WT muscle, whereas satellite cells of uninjured SC-IKK muscle did not differ from those of uninjured WT muscle (n=5, Figure



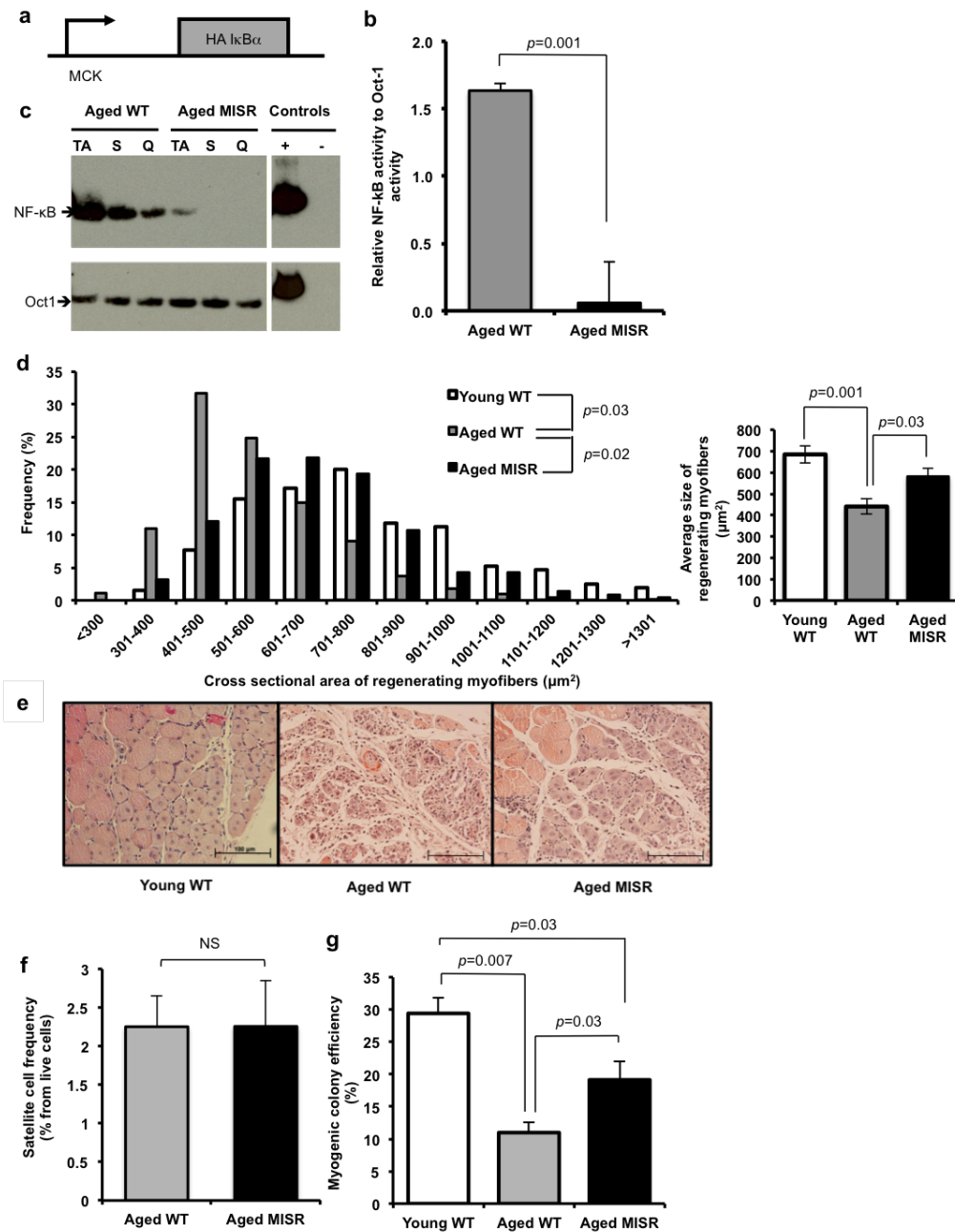
6a). Also, prolonged satellite cell-specific NF- $\kappa$ B activation deteriorated the myogenic function of satellite cells. After 6-9 months of satellite cell-specific NF- $\kappa$ B activation, SC-IKK mice showed a more rapid decline in colony formation efficiency and muscle regeneration after cryoinjury than age-matched WT controls (n=4-5, Figure 6b,c). This result likely reflects the formation of an NF- $\kappa$ B-activated niche over time due to gradual accretion in muscle fibers of IKK-expressing satellite cell nuclei, as satellite cells are spontaneously activated from wear and tear to maintain the muscle homeostasis. This altered niche then acts indirectly to impair satellite cell function in SC-IKK muscle.



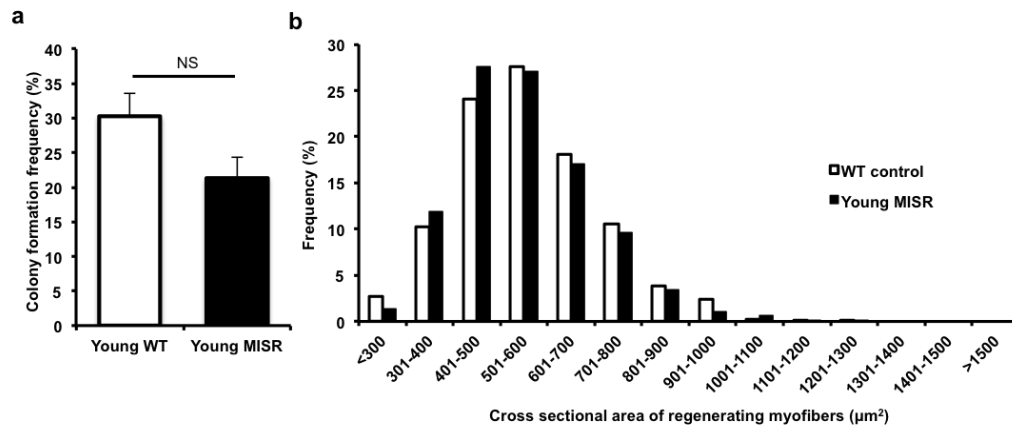
**Figure 6.** NF- $\kappa$ B-activated niche causes decline in satellite cell function and muscle regeneration in young and mid-aged mice. (a) Colony formation efficiency of satellite cells isolated from injured and uninjured muscles of WT and tamoxifen-injected SC-IKK mice was compared. (b) Colony formation efficiency of satellite cells isolated from 1-year old SC-IKK with 6-9 month of NF- $\kappa$ B activation, 1-year-old littermate controls, 2-month-old and 2-year-old WT controls. (c) Skeletal muscle regeneration 7 days after dry ice injury in 1-year-old SC-IKK and 1-year-old and 2-month-old WT was analyzed by cross-sectional area distribution of regenerating myofibers (left) and the average size of regenerating myofiber size (right).

***Muscle-specific inhibition of NF- $\kappa$ B activity improves satellite cell function in an aging model.***

Taken together, the data reported above are consistent with a model in which cell type-specific activation of NF- $\kappa$ B signaling in aged muscle fibers, rather than aged satellite cells, is the primary cause of deficient muscle regeneration in older animals. To further test this notion, I examined satellite cell and muscle regenerative function in aged animals harboring a transgene that constitutively blocks NF- $\kappa$ B signaling in skeletal muscle. Muscle-specific I $\kappa$ B super-repressor (MISR) mice (Figure 7a) constitutively express the I $\kappa$ B super-repressor in skeletal muscle fibers, and have been reported to exhibit nearly complete inhibition of NF- $\kappa$ B activity in mature myofibers of young (8-11 weeks of age) mice (Cai, 2004). I confirmed NF- $\kappa$ B inhibition by the MISR transgene in aged (24-26 months of age) mice by EMSA analysis (n=6, Figure 7b,c). In addition, consistent with a detrimental effect of increased NF- $\kappa$ B activity in aged myofibers on muscle regenerative potential, the cross-sectional area of regenerated myofibers was significantly greater in 2-year old MISR mice as compared to age-matched littermate controls (n=5, Figure 7d,e). Aged MISR mice were not protected from age-related loss of satellite cells, which were similarly reduced in frequency when isolated from MISR animals or from littermate controls (n=5, Figure 7f). However, lifelong blockade of NF- $\kappa$ B activity in muscle fibers did ameliorate the reduction in satellite cell myogenic activity, measured in colony-forming assays of satellite cells isolated from aged MISR muscle (n=6, Figure 7g). No differences were noted in young MISR mice versus age-matched littermate controls with regard to skeletal muscle regeneration or colony formation (n=7, Figure 8a,b). Thus, modulating inflammatory gene expression in mature muscle fibers yields a cell-non-autonomous effect on resident satellite cells, consistent with a “niche” effect of aging on satellite cell function.



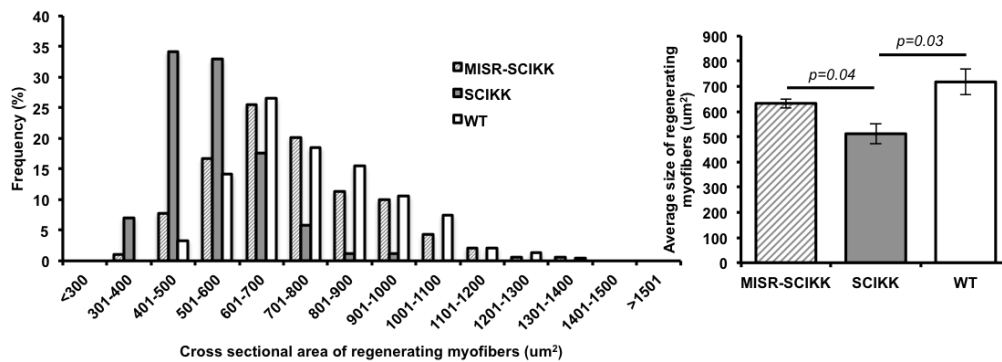
**Figure 7.** Life-long inhibition of NF- $\kappa$ B-activity restores satellite cell function. (a) MISR mice carry a constitutively active, Hyaluronic Acid (HA) tagged, I $\kappa$ B gene under control of the muscle- specific MCK promoter, and thus exhibit diminished NF- $\kappa$ B activity in a skeletal muscle specific fashion. (b) Representative EMSA, quantified in (c), demonstrating minimal NF- $\kappa$ B activity in the hind limb muscle of aged MISR mice. (d) Aged MISR mice exhibit increased size of regenerating myofibers at day 7 following dry ice injury. (e) Representative H&E images of skeletal muscle sections taken 7 days after cryoinjury and stained with H&E demonstrate robust repair in young mice, a decreased regenerative response in aged mice, and improved repair in similarly aged MISR mice. (f) Frequency of satellite cells (percent of live cells by flow cytometry) is not different between aged MISR mice versus Aged WT. (g) Double sorted satellite cells from aged MISR mice demonstrate significantly increased myogenic colony-forming efficiency as compared to age matched WT mice, although colony formation is still reduced as compared to satellite cells from young WT mice. MISR mice were allowed to age alongside age-matched wild-type controls for these studies. Scale bars represent 100  $\mu\text{m}$ . Young mice ranged in age from 8-11 weeks. Aged mice ranged in age from 24-26 months. All figures represent mean  $\pm$  SEM.



**Figure 8.** Satellite cell function and skeletal muscle regeneration in young MISR mice. (a) Young WT and young MISR mice show no difference in the myogenic colony formation efficiency. (b) Skeletal muscle regeneration at 7 days after dry ice injury was similar between young WT and MISR.

### ***Muscle regeneration is restored in MISR-SCI-KK mice***

Next, I generated MISR-SCI-KK mice by crossing MISR and SCI-KK mice to test if dominant inhibition of NF-κB in myofibers alone is sufficient to restore the impaired muscle regeneration in SC-IKK muscle. As shown in Figure 9, MISR-SCI-KK mice indeed showed improved muscle regeneration compared to SCI-KK littermates as measured by the cross-sectional area of regenerating myofibers 7 days after dry ice injury (n=5, Figure 9).



**Figure 9.** Improved muscle regeneration in MISR-SCI-KK mice. Cross sectional area of regenerating myofibers in MISR-SCI-KK mice were compared to age-matched SC-IKK and WT mice at 7 days after cryoinjury. Size distribution (left) and the average size of regenerating myofibers (right) show impaired muscle regeneration in SC-IKK mice is restored in MISR-SCI-KK mice.

***Sodium salicylate treatment improves myogenic function of satellite cells in aged mice and SC-IKK mice.***

Thus far, my data in two complementary transgenic mouse models demonstrates a non cell-autonomous inhibitory effect of increased NF- $\kappa$ B stimulated gene expression on satellite cell function. Additionally, my data from MISR animals suggests that restraining NF- $\kappa$ B activity throughout life may protect against some age-acquired defects in satellite cell activity and muscle regeneration (Figure 10). I also wondered whether acute inhibition of NF- $\kappa$ B signaling would also be effective in reversing age-related defects in muscle repair. To test this possibility and evaluate the therapeutic potential of NF- $\kappa$ B inhibition for stimulating muscle repair in aged individuals, I attempted to rejuvenate aged muscle using sodium salicylate, a non-steroidal anti-inflammatory drug previously reported to inhibit NF- $\kappa$ B signaling. As an active metabolite of aspirin, salicylic acid is known to suppress the activity of cyclooxygenase (COX), an enzyme that is responsible for the production of pro-inflammatory mediators such as the prostaglandins. Aspirin and sodium salicylate specifically inhibit IKK $\beta$ /NF- $\kappa$ B axis *in vitro* and *in vivo* by reducing ATP binding in IKK $\beta$  (Yin, 1998). Previous studies have demonstrated high-dose salicylate therapy to be safe in mice and humans and effective in reducing NF- $\kappa$ B mediated inflammation by inhibiting the kinase activity of IKK $\beta$  (Kopp, 1994; Cai, 2004; Goldfine, 2010). Administration of high-dose salicylate also reduced circulating levels of C-reactive protein, nitric oxide, and sCD140L, which are regulated by NF- $\kappa$ B (Goldfine, 2010). The decreases in levels of circulating proteins and enzymatic products are consistent with inhibition of NF- $\kappa$ B and support it as a molecular target of high-dose salicylate. Moreover, systemic administration of sodium salicylate decreased muscle atrophy in MIKK mice caused by myofiber-restricted over-expression of IKK $\beta$  (Cai, 2004). However, what role, if any, sodium salicylate might play in modulating skeletal muscle regeneration has not been studied.

To test salicylate effects on aged muscle satellite cells and muscle regenerative activity,

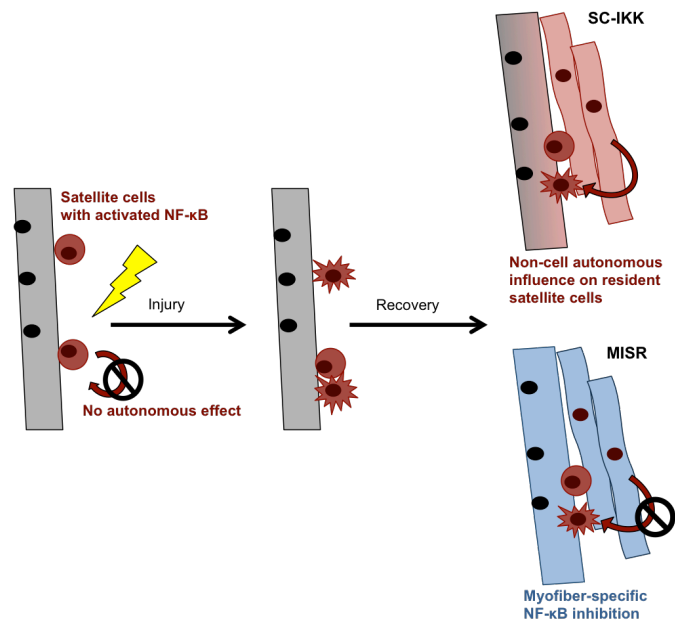
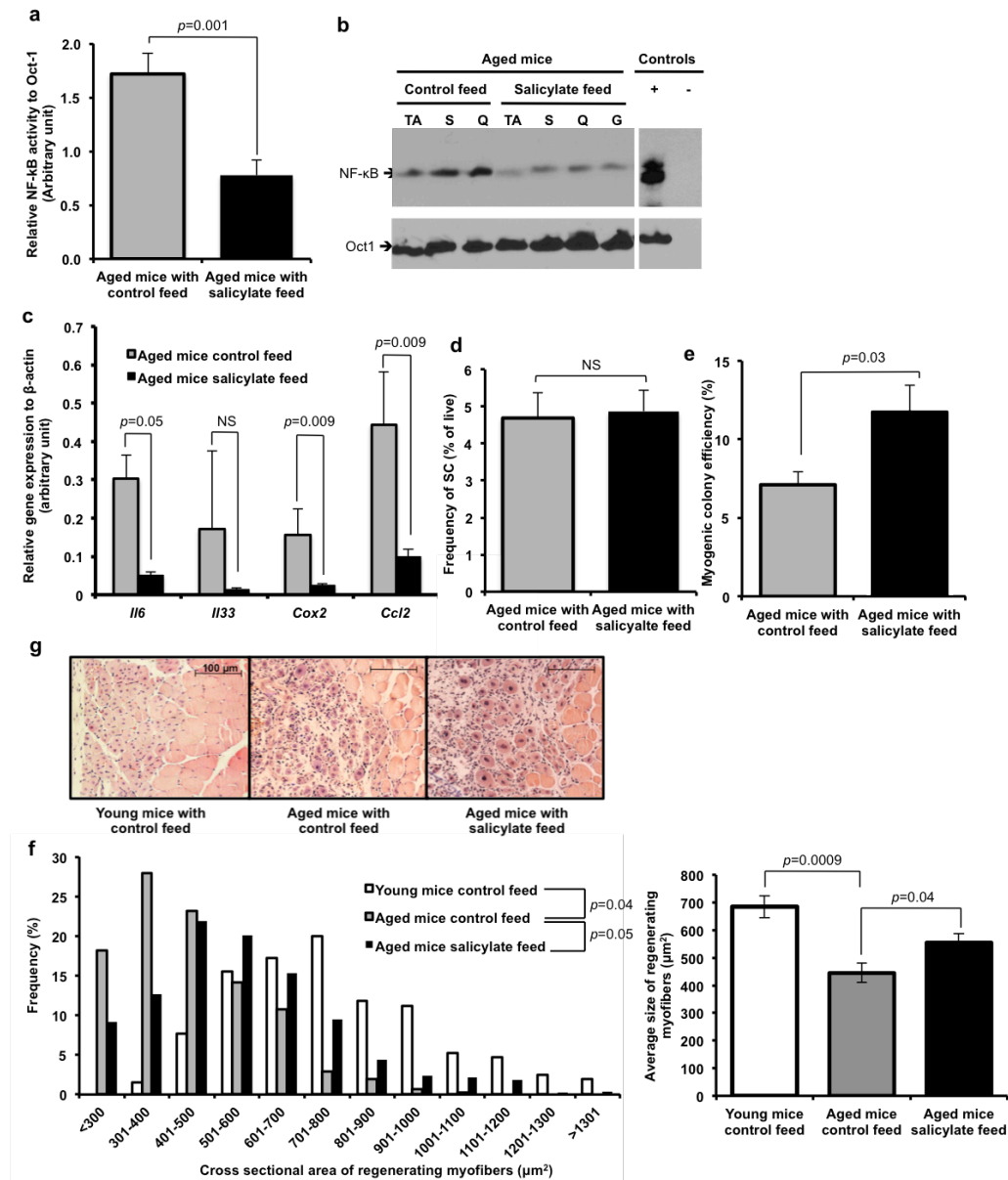


Figure 10. Non cell-autonomous effect of NF-κB in myofibers

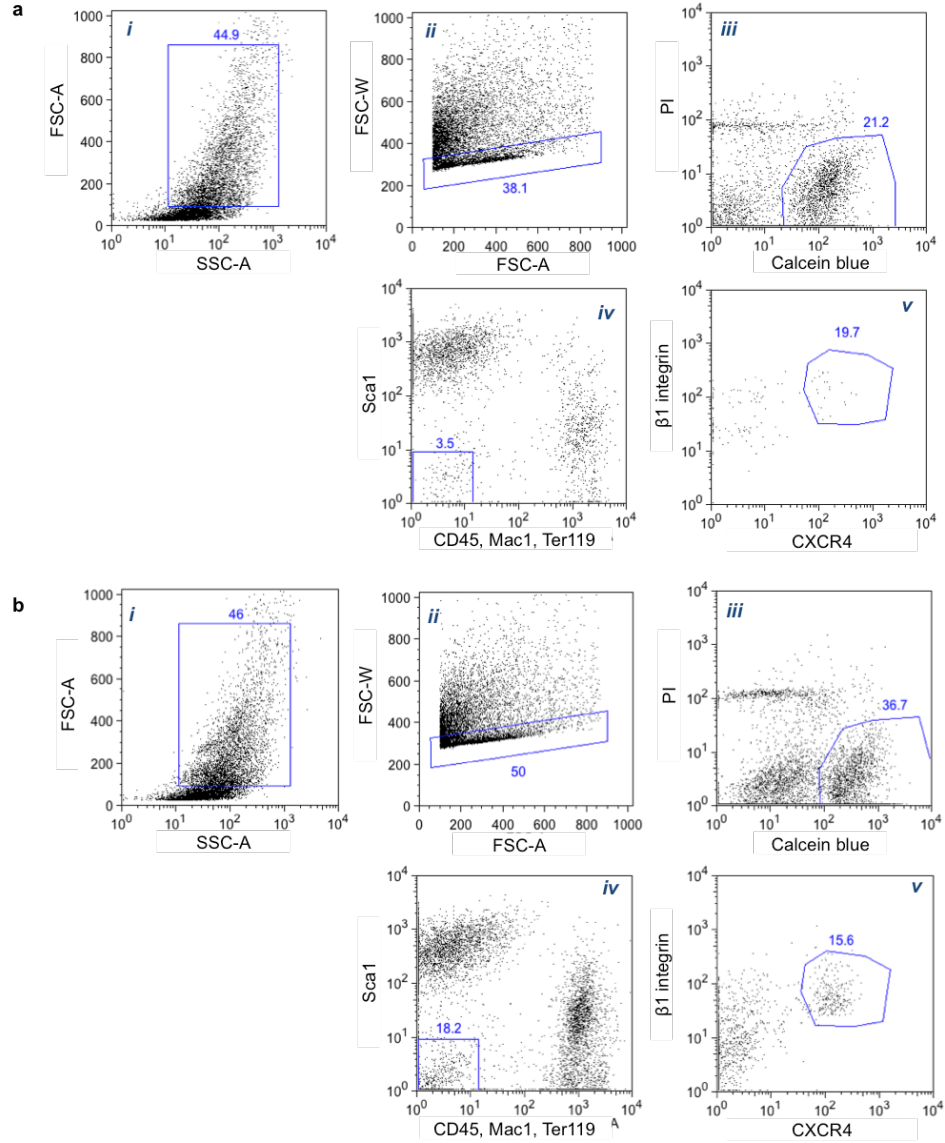
aged mice (24 months of age) were placed on either high-dose sodium salicylate or control diet for a total of 6 to 8 weeks. EMSAs performed on whole hind limb skeletal muscle demonstrated a 50 % decrease in NF- $\kappa$ B activity in salicylate-treated animals (n=9, Figure 11a,b). Freshly isolated satellite cells from sodium salicylate treated aged mice similarly displayed evidence of reduced NF- $\kappa$ B signaling, indicated by a significant decrease in inflammatory gene expression (n=4-6, Figure 11c). Similar to results obtained in MISR mice, satellite cells collected from salicylate treated aged mice also showed no change in satellite cell frequency (n=8, Figure 11d , 12a,b) but did exhibit significant improvement in myogenic colony forming ability (n=8, Figure 11e). Aged mice treated with sodium salicylate also displayed improved muscle regeneration on day 7 following injury *in vivo* (n=7, Figure 11f,g). By day 14, there was a trend towards improved muscle regeneration (n=5, Figure 13a,b). Young mice treated with sodium salicylate versus control feed showed no difference in skeletal muscle regeneration following injury, in satellite cell frequency, or in myogenic colony formation (n=5-7, Figure 14a,b, 15a,b).

In young SC-IKK mice, systemic treatment with the NF- $\kappa$ B inhibitor sodium salicylate rescued the enhanced inflammatory gene expression in tamoxifen-treated SC-IKK satellite cells (Figure 15c), and ameliorated the regenerative defect (n=7, Figure 15d,e), confirming the involvement of heightened NF- $\kappa$ B activity in the observed phenotype. Administration of sodium salicylate did not affect satellite cell frequency or colony-formation of SC-IKK satellite cells (n=5, Figure 15a,b), which were not altered in comparison to age-matched WT controls.

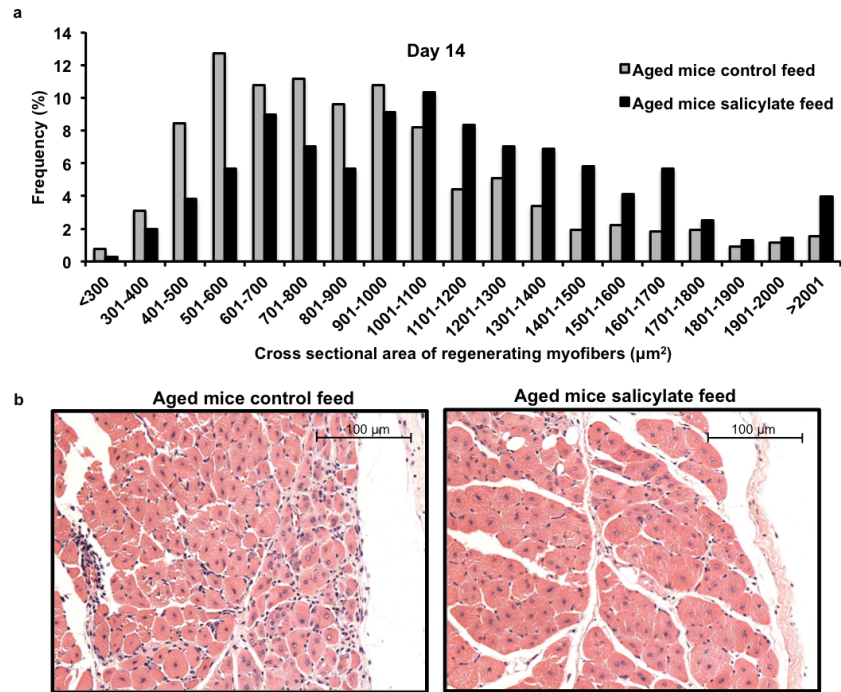


**Figure 11.** Reduction of NF-κB activity by sodium salicylate treatment improves myogenic function of aged satellite cells. (a) Aged mice treated with sodium salicylate demonstrate decreased NF-κB activity in hind limb skeletal muscle as compared to age-matched controls by EMSA. Representative EMSA data are shown in (b). (c) qRT-PCR analysis of sorted satellite cells from aged mice demonstrates a corollary decrease of inflammatory gene expression related to the NF-κB pathway with sodium salicylate treatment. (d) Frequency of satellite cells (percent of live cells by flow cytometry) is not different in aged mice treated with control feed versus those receiving salicylate feed. (e) Double sorted satellite cells from aged mice on salicylate feed have higher rate of myogenic colony formation as compared to age matched mice receiving control feed. (f) Sodium salicylate treatment of aged mice increases the size of regenerating myofibers at days 7. (g) Representative H&E images of skeletal muscle tissue sections taken 7 days after cryoinjury demonstrates robust repair in young mice, a decreased response in aged mice, and improved repair in aged mice having undergone treatment with sodium salicylate. Scale bars = 100 μm. All figures represent mean ± SEM.

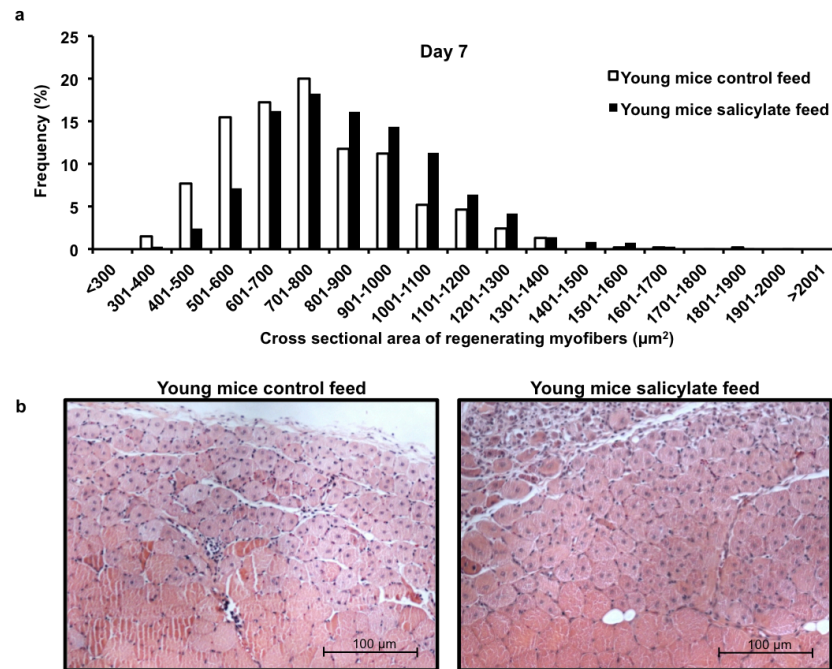




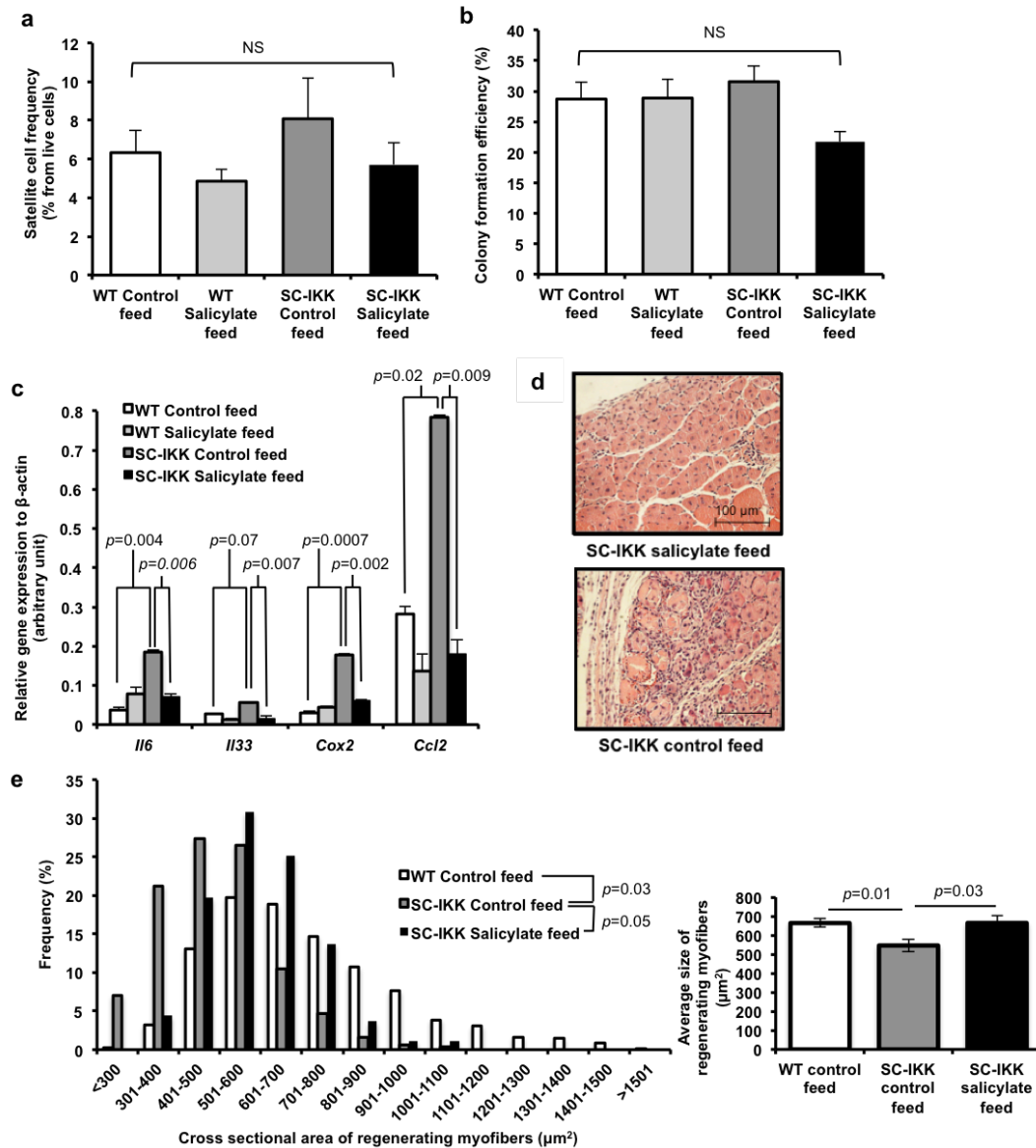
**Figure 12.** Frequency of satellite cells within the myofiber-associated cell population is similar in aged mice treated with either control or sodium salicylate feed. Representative flow plots depict the sequential gating strategy (*i, ii, iii, iv, v*) utilized and in aged mice with control feed (**a**) and salicylate feed (**b**). Numbers represent percentage of previously gated population. Sodium salicylate treatment in aged mice does not change the frequency of CD45-Sca1-Mac1-CXCR4+β1integrin+ satellite cells. Aged mice ranged in age from 23-26 months.



**Figure 13.** Skeletal muscle regeneration in aged mice with or without sodium salicylate treatment at days 14 after injury. (a) Aged mice with salicylate treatment showed a trend of increased regenerating myofibers size up to day 14 after dry ice injury, although it didn't reach the level of statistical significance. (b) Representative pictures of skeletal muscle regeneration at 14 days after dry ice injury in aged mice with or without salicylate treatment. Scale bars = 100 μm.



**Figure 14.** Skeletal muscle regeneration in young mice with or without sodium salicylate treatment at days 7 after injury. (a) Salicylate treatment in young WT does not cause significantly increased regenerating myofiber sizes. (b) Representative pictures of regenerating muscles of young WT mice with control feed or salicylate feed at 7days after dry ice injury. Scale bars = 100  $\mu\text{m}$ .



**Figure 15.** Sodium salicylate treatment in SC-IKK mice restores muscle regeneration. (a) The frequency of satellite cells and (b) satellite cell myogenic colony formation efficiency remained unperturbed in SC-IKK mice and WT mice after salicylate treatment. (c) Satellite cells from SC-IKK mice demonstrate increased expression of NF- $\kappa$ B target genes following tamoxifen treatment. Sodium salicylate treatment restored expression of these genes in satellite cells from SC-IKK mice to wild-type levels. (d,e) Impaired SC-IKK mice was restored by salicylate treatment. Muscle regeneration was assessed by H&E staining at 7 days after cyroinjury and compared to age-matched WT controls and SC-IKK with or without salicylate treatment. Scale bars = 100  $\mu\text{m}$ . All data are presented as mean  $\pm$  SEM.

***Satellite cells with NF- $\kappa$ B activation exhibit increased DNA damage that is reversed by inhibition of NF- $\kappa$ B activity in myofibers.***

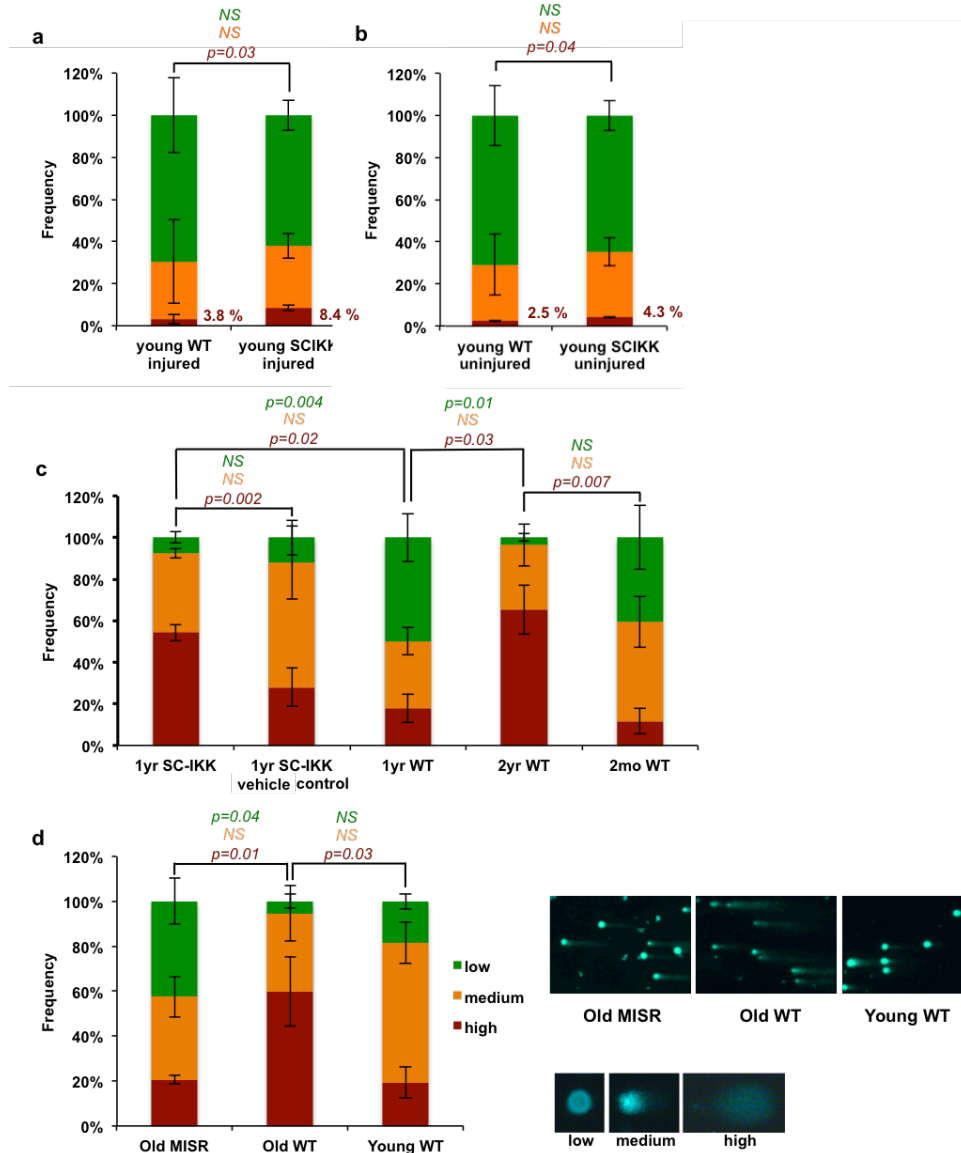
Next question I asked was about how NF- $\kappa$ B activation causes impairments in satellite cell function. NF- $\kappa$ B has been implicated in cellular senescence and oxidative damage, including DNA damage and protein damage through the generation of mitochondrial reactive oxygen species. As discussed above, stem cells persist throughout life in a largely quiescent state. As a result, stem cells in aged tissues experience long-term exposure to genotoxic assaults, from both endogenous and exogenous sources, and an apparent accumulation of DNA damage in aged stem cells has been noted in several studies. For example, aged HSCs and satellite cells show an increased number of nuclear foci that stain for the phosphorylated form of the variant histone H2A.X (gH2A.X), which serves as a marker of DNA double-strand breaks (Rossi, 2007; Rübe, 2011; Sinha, 2014; Rogakou, 1998). Accumulation of DNA damage in these stem cells can also be detected using single-cell gel electrophoresis assays, or comet assay (Sinha, 2014; Beerman, 2014). However, the level of DNA damage in stem cells must be interpreted with caution with regard to its role in aging because such damage can be repaired, or asymmetrically passed on to daughter cells, and may be involved in normal cellular process such as differentiation (Beerman, 2014; Larsen, 2010; Charville, 2011).

Recently, our lab has shown that the high level of DNA damage in satellite cells of aged mice can be reversed by exposure of systemic factors in circulation of young mice (Sinha, 2014). Also, different levels of DNA damage induced by irradiation exhibited negative correlation with satellite function, suggesting the relevance of DNA damage to muscle regeneration (Sinha 2014). Based on these data, I examined DNA damage levels in satellite cells of injured and uninjured SC-IKK muscle. Comet assay on SC-IKK satellite cells showed a greater percentage of cells with highly damaged DNA than the fraction of damaged nuclei seen among WT satellite cells, and this difference was amplified when the muscles were injured (n=4, Figure 16a,b).

These data suggest that both cell-autonomous and non cell-autonomous NF- $\kappa$ B signaling might contribute to the accumulation of DNA damage in satellite cells. Satellite cells of older SC-IKK mice with prolonged NF- $\kappa$ B activation in satellite cells also showed higher DNA damage level compared to age-matched WT controls (n=4-6, Figure 16c). Importantly, life-long inhibition of NF- $\kappa$ B activity in myofibers (aged MISR mice) appeared to prevent the accumulation of DNA damage in aged satellite cells, with the fraction of satellite cells with highly damaged DNA approximately unchanged in aged MISR mice as compared to young controls (n=4, Figure 16d). These data suggest that inhibition of non cell-autonomous NF- $\kappa$ B signaling preserves the myogenic function of aged satellite cells in part by protecting them from DNA damage accumulation. The precise mechanisms by which NF- $\kappa$ B causes accumulation of DNA damage in satellite cells and how that accumulation leads to declining satellite function remains to be studied.

## ***Discussion***

In summary, the data reported in this chapter suggest that chronic inflammation, mediated by NF- $\kappa$ B, retards the myogenic potential of satellite cells in aged muscle. Substantiating this observation, satellite cells from young mice with genetically induced high IKKb activity exhibit poor skeletal muscle regeneration, an effect that can be partly reversed with sodium salicylate treatment. However, although NF- $\kappa$ B target genes were activated in satellite cells in aged mice, and in mice harboring a satellite cell-specific IKKb transgene, these satellite cells exhibited no cell intrinsic defects in frequency or myogenic function. Rather, defective satellite cell function in the context of induced NF- $\kappa$ B activity was revealed only when mature muscle fibers exhibited this heightened NF- $\kappa$ B signaling (either in aged mice or when SC-IKK nuclei were incorporated into regenerating myofibers during muscle repair). These data are consistent with cell extrinsic regulation of satellite cell function in these contexts, a notion that is further supported by the



**Figure 16.** DNA damage induced by activation of NF- $\kappa$ B signaling in satellite cells can be reversed by inhibition of NF- $\kappa$ B activity in muscle. Levels of DNA damage in satellite cell was measured by comet assay. (a,b) DNA damage level in satellite cells isolated from injured or uninjured muscles of SC-IKK and WT mice were measured. Satellite cells from injured SC-IKK muscle exhibited greater number of cells with higher DNA damage level compared to satellite cells from injured or uninjured WT muscles. (c) 1-year-old SC-IKK mice with prolonged NF- $\kappa$ B activation showed higher DNA damage level in satellite cells than age-matched WT, 2-month-old or 2-year-old WT mice. (d) Life-long inhibition of NF- $\kappa$ B activity reversed the higher DNA damage level in aged satellite cells. DNA damage level of satellite cell was assessed by the length of comet formed after single cell electrophoresis and scored low, medium, and large as shown in the bottom right panel of (c). All data are presented as mean  $\pm$  SEM.

observation that aged MISR mice, which maintain dominant inhibition of NF- $\kappa$ B function in muscle fibers alone, are partially protected from age-dependent loss of satellite cell myogenic function. Improved muscle regeneration in MISR-SCIKK mice, with activation of NF- $\kappa$ B in satellite cell and inhibition of NF- $\kappa$ B in myofibers, also adds evidence to support the cell extrinsic regulation of satellite cell function by NF- $\kappa$ B signaling in muscle fibers.

The results reported here are consistent with prior studies demonstrating that NF- $\kappa$ B inhibition can slow muscle atrophy (Li, 2008) and can increase the cross sectional area of regenerating fibers following injury in a murine model of muscular dystrophy (Mourkioti, 2004). However, this study is the first to discriminate the impact of NF- $\kappa$ B elevation in skeletal muscle fibers versus satellite cells, and reveals a surprising non-autonomous regulation of satellite cell regenerative function by NF- $\kappa$ B driven gene expression in regenerating myofibers.

This study further demonstrates that systemic inhibition of NF- $\kappa$ B activity promotes more robust muscle regeneration in aged animals. Administration of high-dose sodium salicylate improves muscle regeneration and should be considered as a potential pharmacologic therapy. In addition, more specific inhibitors of IKK $\beta$  might be useful to develop treatments for sarcopenia, since sodium salicylate itself has a wide ranges of other targets, and the high doses of it required for IKK $\beta$  inhibition are often not well tolerated due to dose-dependent and dose-limiting side effects, such as tinnitus, headache and irritation in gastrointestinal tract. To date, only one other FDA approved drug, Losartan, has shown promise at a basic science level as a potential therapeutic agent (Burks, 2011). Further studies of this pathway will be helpful in revealing other potential targets for improving skeletal muscle regeneration in older age.

As for how satellite cell function is affected by extrinsic NF- $\kappa$ B signaling, this study suggests accumulation of DNA damage induced by activation of NF- $\kappa$ B. The mechanisms by which accumulation of DNA damage in aged stem cells may contribute to stem cell dysfunction are still being elucidated. Genotoxic lesions could cause stem cell senescence or apoptosis and



might directly affect gene regulation, leading to alterations in stem cell self-renewal and differentiation. Elevated levels of damaged DNA in aged stem cells could result from an accumulation of damage over time, an increase in the rate of damage, a decrease in the rate of repair in response to DNA damage, or a combination of these possibilities. Supporting a role for changes in the DNA damage response (DDR), aged human HSC show compromised capacity to repair experimentally introduced DNA damage, such as that produced by ionizing radiation (Rube, 2011). These observations suggest that DDR pathways, evolved to safeguard genomic integrity and maximize survival of the organism (Rossi, 2008; Ciccia, 2010; Fortini, 2013; Behrens, 2014), may show reduced activity in stem cells with age. Indeed, such changes have been observed in other aging cell types (Moskalev, 2013; Bernardes de Jesus, 2013). However, in muscle satellite cells, DDR protein levels did not alter with aging (Shinha, 2014). Sinha et al rather pointed out the conserved necessity of strand break in stem cells for nuclear reprogramming to allow for cell differentiation (Larsen, 2010). Based on the up-regulation of muscle differentiation gene in aged satellite cells, it was suggested that aged satellite cell are arrested at an early stage of myogenic differentiation which induce DNA strand breaks without resolving them (Shinha, 2014). As previous observation implicated NF- $\kappa$ B in regulation of myogenic differentiation as an inhibitor of MyoD expression (Guttridge, 2000), it will be interesting to see if NF- $\kappa$ B activity is involved in arresting aged satellite cells in early stages of myogenic differentiation and inducing unresolved, differentiation-related DNA strand breaks.

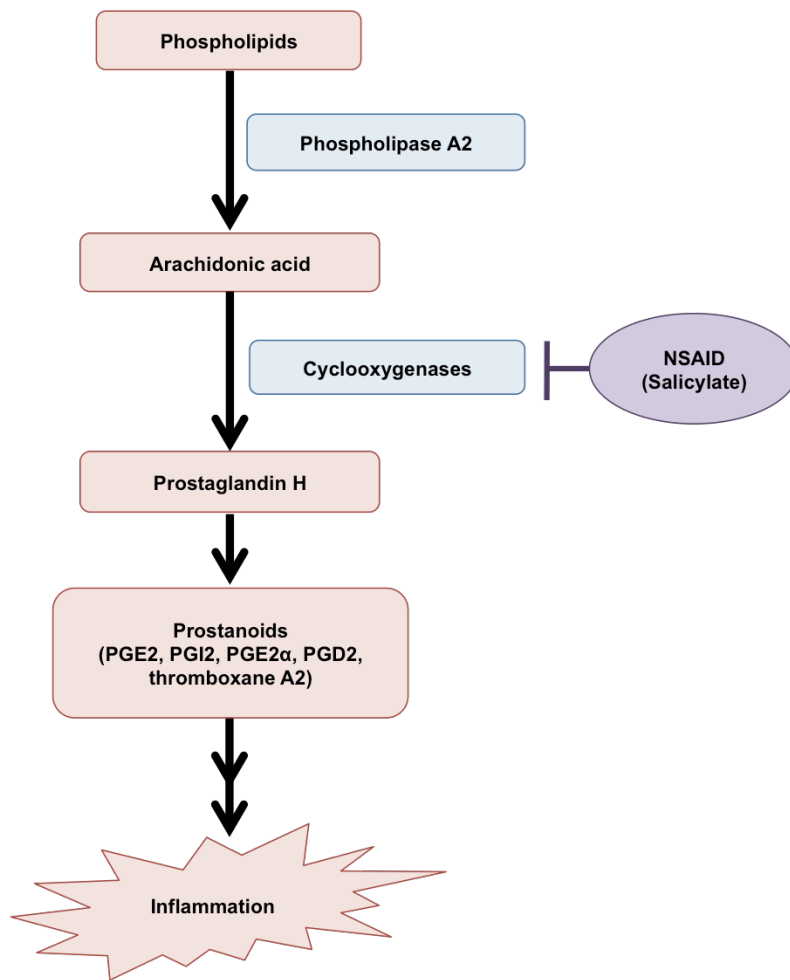
### **Chapter 3.**

## **Secretory Phospholipase A2 Functions as a Downstream Effector of NF- $\kappa$ B-mediated Chronic Inflammation in Aged Muscle.**

## ***Preface***

Secretion of phospholipase A2 (PLA2) is thought to induce inflammatory responses in neighboring cells, initiating or potentiating the local and systemic inflammatory processes such as sepsis and associated acute lung injury as well as inflammatory arthritides (Balsinde, 1999; Ohta, 2013). There are three broad classes within PLA2 superfamily: calcium-independent, secretory, and cytosolic PLA2; and each class is subdivided into isoenzymes. The PLA2 superfamily shares a common feature of catalyzing the hydrolysis of membrane phospholipids to generate lysophospholipids and free fatty acids including arachidonic acid. Arachidonic acid liberated by PLA2 is converted into prostaglandin H2 (PGH2) by catalytic activity of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). Then by various terminal synthases, PGH2 is converted into biologically active prostanoids, including prostaglandins PGE2, PGI2, PGE2 $\alpha$ , PGD2 and thromboxane A2.

Aspirin and most of the widely known non-steroidal, anti-inflammatory drugs currently in use inhibit cyclooxygenases, thereby suppressing the synthesis of prostaglandins (Vane, 1998). As discussed in Chapter 2, treatment of a non-steroidal, anti-inflammatory drug, sodium salicylate, was shown to improve muscle regeneration by enhancing satellite cell function in aged mice, providing an intriguing insight into possible roles of PLA2 and prostaglandins in aging of the muscle stem cell niche (Figure 17). Although the role of secretory PLA2 in skeletal muscle still remains unclear, accumulating evidence suggests that prostaglandins and leukotrienes, another type of metabolite of arachidonic acid, are involved in causing muscular pain and inflammation, and also in myogenesis and the repair of muscles (Korotkova, 2014).



**Figure 17.** Brief schematic of arachidonic acid cascade

A gene array analysis of muscles collected from young WT, aged WT, and aged MISR mice that we performed in an effort to identify possible secretory factors that may be involved in the NF- $\kappa$ B-regulated influence of the aged muscle niche on satellite cells, revealed pla2g5 and pla2g7 (genes that encode PLA2 group V and VII respectively), as promising candidate mediators of this effect (Supplemental figure 3, 4, 5, Supplemental table 1). Validation of our gene array results by qRT-PCR analysis confirmed a number of candidate genes that were up-regulated in aged WT but down-regulated in aged MISR, young WT and young MISR muscle

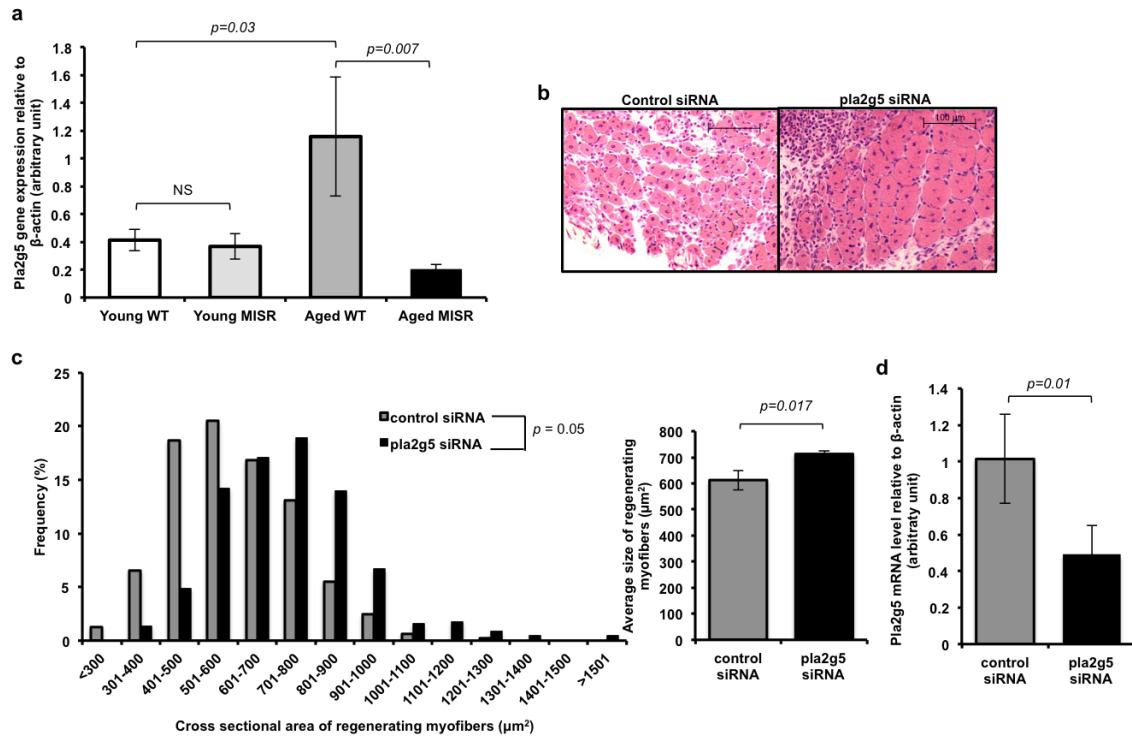
(n=5-6, Supplemental figure 4). To test if any of the validated genes have a potential role in muscle regeneration in aged mice, I divided them into two groups and performed pooled screens by electroporating cocktails of siRNAs for each group of genes into aged TA muscles. At 24 hours post electroporation, muscles underwent cryoinjury and were harvested at day 7 after injury. Knockdown of one group of genes (kcnab1, pla2g5, pla2g7, and cadm1) showed a trend towards increased cross sectional area of regenerating myofibers in the cryoinjured TA muscle of aged mice (Supplemental figure 5), driving our further interest in sPLA2s and their role in the regenerative biology of aged muscle.

## **Results**

### ***Knockdown of pla2g5 improves muscle regeneration in aged mice.***

Pla2g5 encodes PLA2 group V, a member of the calcium-dependent, secretory PLA2 family. Transcription factor binding site analysis by Genomatix (Supplemental table 1) and ChIPseq data provided by the genome browser of The Encyclopedia of DNA Elements (ENCODE) showed NF- $\kappa$ B binding site in the promoter region of pla2g5 gene (data not shown), corroborating the regulation of pla2g5 by NF- $\kappa$ B. High affinity binding of NF- $\kappa$ B to the promoter region of pla2g5 was also witnessed by protein-DNA mobility shift assays in a recent study focused on pulmonary inflammation (Sun, X., 2014).

Expression of pla2g5 is increased in aged skeletal muscle as compared to young WT, young MISR or aged MISR mice (n=6, Figure 18a). Furthermore, inhibition of pla2g5 expression by *in vivo* electroporation of pla2g5 siRNA was sufficient to restore skeletal muscle regeneration in aged mice (n=5, Figure 18b,c), suggesting that NF- $\kappa$ B inhibits muscle regeneration in part by up-regulation of pla2g5 within skeletal muscle fibers.



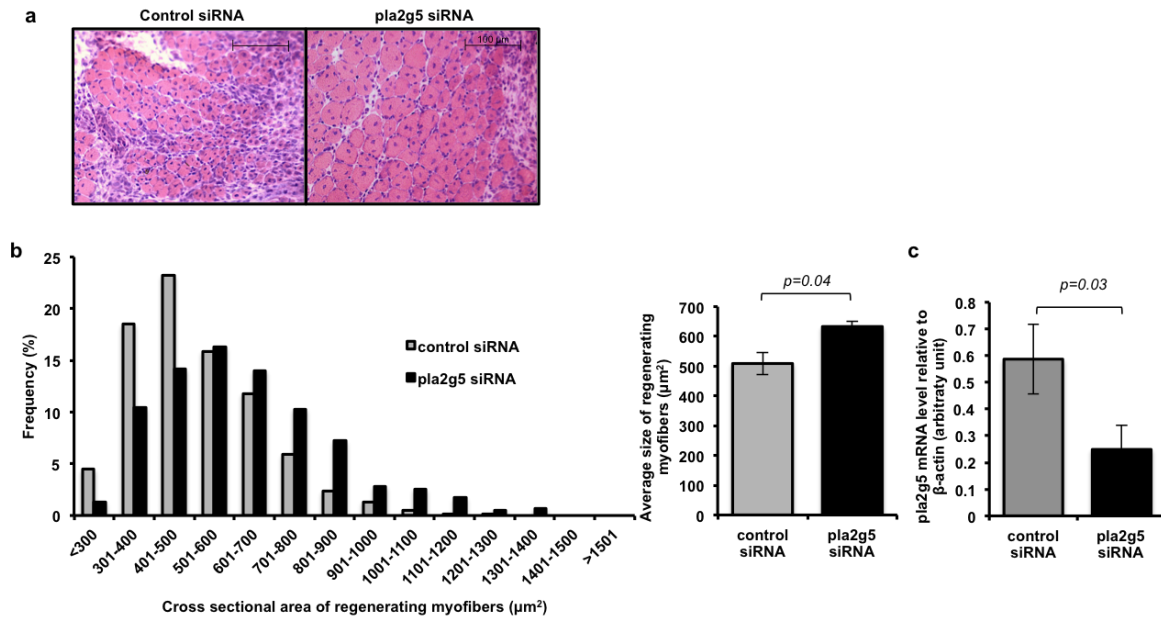
**Figure 18.** Knockdown of pla2g5 improves muscle regeneration in aged mice. (a) mRNA level of pla2g5 measured by pRT-PCR in the whole muscle tissue of young and aged WT and MISR mice. (b) Representative H&E staining of frozen sections of electroporated aged muscle at 7 days after dry ice injury. (c) Improved muscle regeneration with electroporation of pla2g5 siRNA in aged muscle analyzed by size distribution and average size of regenerating myofibers. (d) Knockdown of pla2g5 gene expression was validated by qRT-PCR at the harvest. Pla2g5 gene expression was decreased by approximately 52 %. Scale bars = 100  $\mu\text{m}$ . All data are presented as mean  $\pm$  SEM.

The level of gene knockdown in aged muscles electroporated with pla2g5 siRNA was measured at the time of tissue harvest and was 51.9% on average (S.D $\pm$ 24.1%, n=14) (Figure 18d). This knockdown level was comparable to the difference in the level of pla2g5 mRNA in aged WT versus aged MISR or young WT, as shown in Figure 17a. For controls, contralateral TA muscle was electroporated with scrambled siRNA designed to have no specificity to any mammalian genes but having a similar GC content as pla2g5 siRNA. mCherry fluorescent protein-expressing plasmid was co-electroporated with the siRNA as a reporter of fiber transduction, allowing for estimation of the efficiency of electroporation by visualizing mCherry<sup>+</sup> myofibers in each sample (Supplemental figure 6).

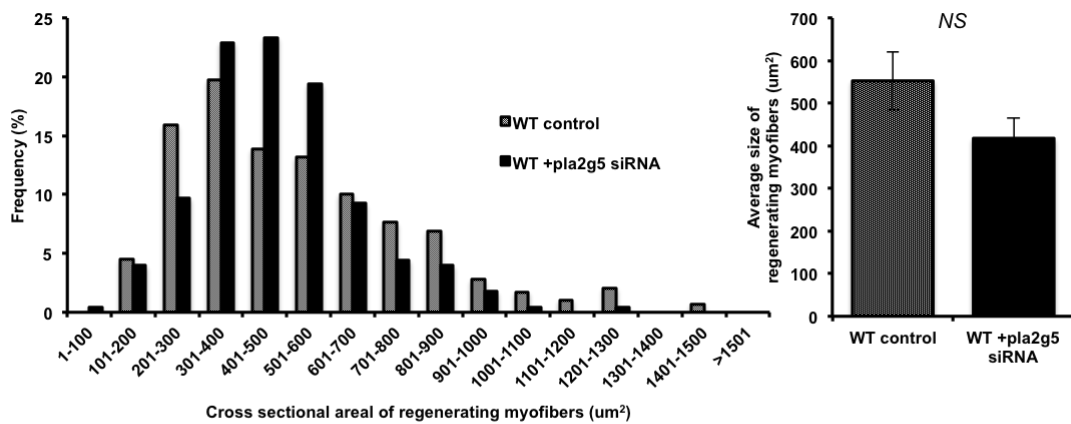
I also performed a linear regression analysis between knockdown level of pla2g5 expression and increase in average size of regenerating myofibers, to evaluate whether knockdown of pla2g5 improves muscle regeneration in a dose-dependent manner. A trend of increasing regenerating myofiber size with higher knockdown level was observed, although it did not reach statistical significance (n=14, Supplemental figure 7). Thus, PLA2G5 may not necessarily behave in a dose-dependent manner in muscle regeneration, possibly because there is a threshold level of PLA2G5 required for its pro-inflammatory role that affects muscle regeneration in aged mice. Also, it is possible that translational or post-translational regulation of pla2g5 mRNA or protein also impacts the outcome of pla2g5 knockdown in muscle regeneration.

***Knockdown of pla2g5 restores NF- $\kappa$ B-mediated impairment of muscle regeneration.***

To test whether the beneficial effect of knocking-down pla2g5 in muscle regeneration is specific for NF- $\kappa$ B-induced muscle inflammation, tamoxifen-induced SC-IKK mice (3-months old) were treated with pla2g5 siRNA electroporation in muscle and analyzed for their regenerative capacity under the same conditions under which old mice were examined (see above). SC-IKK mice also showed improved muscle regeneration when electroporated with pla2g5 siRNA prior to cryoinjury (n=5, Figure 19a,b), with an average 64.3% (S.D $\pm$ 27.3) knockdown of pla2g5 expression (Figure 19c). Such improvement in muscle regeneration was not present in age-matched young WT controls in which pla2g5 was knocked down (Figure 20).



**Figure 19.** Improved muscle regeneration in SC-IKK mice with knockdown of pla2g5. (a) Representative H&E staining of frozen sections of SC-IKK muscle electroporated with either pla2g5 siRNA or control siRNA. (b) Size distribution or average size of regenerating myofibers of SC-IKK muscle with pla2g5 siRNA electroporation show improved muscle regeneration with knockdown of pla2g5 gene expression. (c) pRT-PCR analysis of pla2g5 gene expression showed approximately 64% of knockdown in SC-IKK mice. Scale bars = 100 μm. All data are presented as mean ± SEM.



**Figure 20.** Knockdown of pla2g5 does not affect muscle regeneration in young WT mice. Muscle regeneration assessed by size distribution and average size of regenerating myofibers in young WT muscles with or without pla2g5 siRNA show no difference. All data are presented as mean ± SEM.



## ***Discussion***

This study establishes PLA2G5 as a novel downstream effector of NF- $\kappa$ B in regulating muscle repair in response to injury, and demonstrates that inhibition of this target in muscle fibers alone is sufficient to reverse age-related and NF- $\kappa$ B-mediated deficits in satellite cell activity and function. Further studies are required to understand at a molecular and biochemical level how PLA2G5 alters satellite cell physiology to influence muscle regeneration.

PLA2G5 catalyzes the hydrolysis of membrane phospholipids to generate lysophospholipids and free fatty acids including arachidonic acid. It preferentially hydrolyzes linoleoyl-containing phosphatidylcholine substrates. Secretion of this enzyme is thought to induce inflammatory responses in neighboring cells. This enzyme, once secreted, associates with the outer surface of the cells and then releases arachidonic acid, which can be captured by surrounding cells to produce eicosanoids, including the prostaglandins and its analogs, thromboxanes, lipoxins, and leukotrienes, (Balboa, 1996; Balsinde, 1999). Based on these previous observations, I postulate that PLA2G5 secreted by myofibers into the interstitial space in muscle tissue react with membrane phospholipids of neighboring myofibers, and many other cell types that are present in muscle tissue, such as fibroblasts, endothelial cells and smooth muscle cells. Several studies have convincingly demonstrated that skeletal myofibers can produce prostaglandins. For example, isolated primary chick myoblasts and myotubes are able to produce PGE and PGF in vitro from endogenous arachidonic acid, and this process is enhanced in the presence of exogenous arachidonic acid (McElligott, 1988). Also, human and mouse myoblasts produce PGE<sub>2</sub> and PGF<sub>2</sub> $\alpha$  (Zalin, 1987; Otis, 2005), and primary mouse myoblasts express PGI synthase and the PGI<sub>2</sub> receptor and generate PGI<sub>2</sub> (Bondesen, 2007).

Activation of PLA2 is a rate-limiting step in the biosynthesis of prostaglandins (Cirino, 1998). Although little is known about PLA2 expressed and secreted by skeletal muscle tissue, a role for different types of prostaglandins in myoblasts has been well documented. In studies

published to date, prostaglandins have been reported to influence multiple steps of myogenesis, including proliferation (Zalin, 1987; Otis, 2005), differentiation (Zalin, 1987), migration (Bondesen, 2007), fusion (Bondesen, 2007), and survival of myoblasts (Jansen, 2008).

On the other hand, prostaglandins are powerful mediators of inflammation and pain, and are involved in cytokine production and nitric oxide synthase expression, increases in vasodilation and vascular permeability, and chemotaxis of inflammatory cells. PGE<sub>2</sub>, in particular, seems to be a particularly potent mediator of muscular pain and inflammation, and has been suggested as a potential mediator of skeletal muscle wasting (Tegeder, 2002; Hedenberg-Agnusson, 2001). However, controversy exists regarding the effect of PGE<sub>2</sub> on protein breakdown in isolated skeletal muscles (Rodemann, 1982a; Rodemann, 1982b; Barnett, 1987; Hasselgren, 1990). A number of animal models of diseases have linked excessive production of prostaglandins to muscle wasting (Rieu, 2009; Granado, 2007; Strelkov, 1989; McCarthy, 2004). In a rat model of arthritis, muscle wasting was associated with the induction of specific E3 ubiquitin ligating enzymes, such as muscle atrophy F-box and muscle specific RING finger protein 1 (MuRF1), which are markers of muscle atrophy (Strelkov, 1989). PGE<sub>2</sub> directly induced the expression of these mediators of muscle loss in human skeletal muscle *ex vivo*, thereby confirming its role in muscle wasting (Standley, 2013). Whether the PGE<sub>2</sub> biosynthetic pathway is up-regulated in the muscles of patients with chronic inflammatory diseases is, however, unknown. Regardless, PGE<sub>2</sub> appears to be a promising candidate to study as a mediator of PLA<sub>2</sub>G<sub>5</sub>-driven decline in muscle function. As prostaglandins function as both autocrine and paracrine signal, it is possible that prostaglandins produced in myofibers further propagate the pro-inflammatory effect of PLA<sub>2</sub>G<sub>5</sub> to neighboring cells in muscle tissue.

In addition, the role of this secreted phospholipase in age-related deterioration of muscle regeneration might lead to an interesting insight into a previously unappreciated role of lipid mediators in muscle function. A recent study by Sato et al reported that adipocyte-induced

PLA2G5 drives M2 polarization of macrophages in adipose tissue of obese mice (Sato et al, 2014). As M1 and M2 macrophages have distinct roles at different stages of muscle regeneration, studies to examine the resident M1 and M2 populations in muscles of young and aged mice, and mice with different modulation of NF- $\kappa$ B or pla2g5 activity, might provide a new aspect in the role of muscle-derived PLA2G5 in aging skeletal muscle.

## **Chapter 4.**

### **IL-6 restricts satellite cell function**

## ***Preface***

Interleukin-6 (IL-6) is a pleiotrophic cytokine that plays a key role in inflammation by modulating a variety of physiological responses and activating genes associated with cellular proliferation, differentiation, and apoptosis (Kishimoto, 2006; Jones, 2005). IL-6 signals through IL-6 receptor and plasma membrane receptor complex, which contains the common signal transducing receptor chain, glycoprotein130. Activation of gp130 leads to the activation of JAK tyrosine kinases, induction of tyrosine phosphorylation and recruitment of signal transducer and activator of transcription 3 (STAT3).

IL-6 is produced by a variety of cell types, including monocytes, fibroblasts, vascular endothelial cells, and skeletal muscle fibers (Akira,1993, Jonsdottir, 2000). As a “myokine”, a cytokine produced from muscle fibers, serum IL-6 levels acutely increase during muscle contraction (Febbraio, 2002a). IL-6 gene expression also increases during functional overload and recovery from disuse atrophy (Whites, 2009; Washington, 2010), indicating IL-6 as a muscle mitogen that can activate cell proliferation in skeletal muscle (Cantini, 1995). On the other hand, recent studies showed that IL-6-activated STAT3 signaling promotes myogenic progression by regulating MyoD in satellite cells (Tierney, 2014).

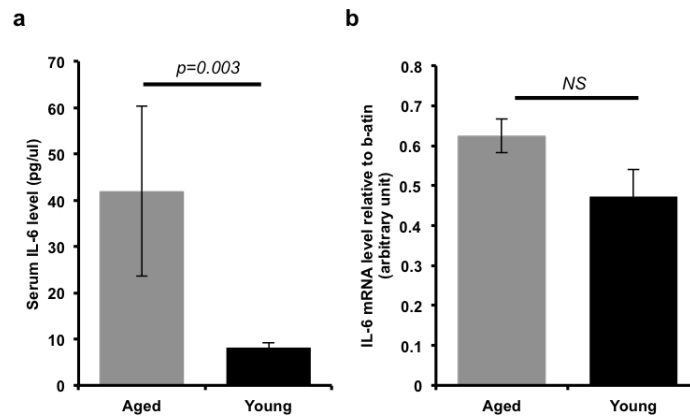
Increased levels of IL-6 have been implicated in numerous pathophysiological conditions, such as cancer cachexia, sepsis, insulin resistance, and acidosis where muscle atrophy is evident (Strassmann, 1992; Baltgalvis, 2008a; Kern, 2001), to induce skeletal muscle protein breakdown (Goodman, 1994). Additionally, a striking correlation exists between age-related muscle defects and chronic inflammation in elderly patients, as measured by high levels of IL-6 (Schaap, 2006). Population studies have associated higher circulating IL-6 with physical frailty and disability, including sarcopenia (Maggio, 2006). In fact, localized chronic IL-6 exposure has been found to cause arrested skeletal muscle growth and induces atrophy in rats (Bodell, 2009;

Haddad, 2004). Also, transgenic mice over-expressing IL-6 at high levels in all tissues and in circulation also exhibit muscle atrophy (Tsujioka, 1995). Based on these observations, I hypothesized that circulating IL-6 is an “aging factor” whose level gradually increases during aging, causing accumulation of damage in cells and degeneration of tissue. The IL-6 gene has a direct binding site for NF- $\kappa$ B, which I found could induce deterioration of satellite cell function and muscle regeneration in aged muscle by mediating chronic inflammatory signaling in muscle fibers (Chapter 2). Moreover, IL-6 was one of the most highly up-regulated target genes of NF- $\kappa$ B expressed by aged in comparison to young satellite cells (Figure 3). These data suggest the potential involvement of IL-6 in regulating satellite cell-mediated muscle regeneration and maintenance in an age-dependent manner.

## **Results**

### ***Serum level of IL-6 is higher in old mice compared to young mice, but IL6 mRNA is not induced in skeletal muscle.***

First, I compared IL-6 expression in young and aged mice at multiple levels. Serum IL-6 level measured by ELISA was significantly higher in aged mice (n=6), verifying a previous study in humans that reported a clear increase in serum IL-6 with advancing age (Young, 1999; Ersler, 1993). Whole muscle IL-6 mRNA levels were higher in aged mice than in young mice, although the difference did not reach statistical significance (n=20, p-value <0.058; Figure 21). Multiple human studies also failed to detect changes in IL-6 mRNA expression in the whole skeletal muscle of elderly humans compared to younger adult muscle (Hamada, 2005; Trenerry M.K., 2008; Przybyla. B., 2006), implying that muscle is not a major source of age-dependent increases in circulating IL-6. In the case of satellite cells, however, IL-6 mRNA level increased with age as shown in chapter 2 (Figure 3e). In addition, a gene array analysis performed previously in the Wagers lab on satellite cells isolated from young, mid-aged, and



**Figure 21.** Expression of IL-6 level in aged and young mice. (a) Serum IL-6 level was measure by IL-6 ELISA in serum collected from young and aged mice. (b) Gene expression level of IL-6 in whole muscle tissue of young and aged mice. All data are presented as mean  $\pm$  SEM.

aged mice indicated IL-6 as one of the top candidate genes whose mRNA level increases the most with age (Supplemental figure 8). This led us to speculate that up-regulation of IL-6 mRNA in satellite cells, together with the systemic elevation of IL-6 protein, might be involved in age-dependent functional decline of muscle regenerative potential.

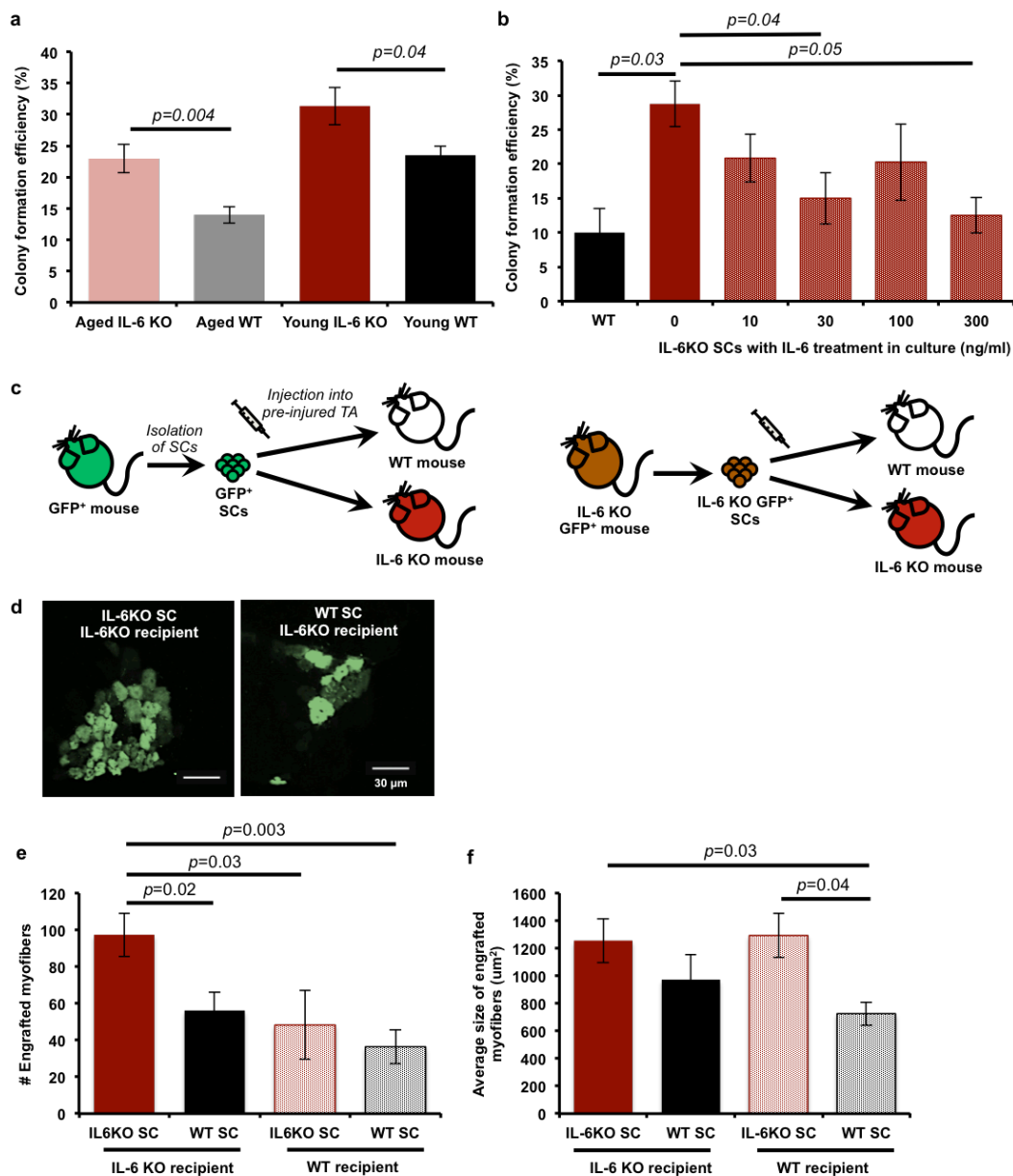
### ***Depletion of IL-6 improves satellite cell survival.***

Based on the results in Figure 20 and on previous studies interrogating the possible role of systemic factors in aging of muscle stem cell (Conboy, 2005), IL-6 knockout (KO) mice with neomycin resistance cassette insertion in the first coding exon, exon 2, of the IL-6 gene were utilized to study the possible role of IL-6 in aging muscle stem cells (Kopf, 1994). Satellite cells isolated from IL-6 KO mice showed restrictive effects of IL-6 on the survival of satellite cells *in vitro*. IL-6 KO satellite cells exhibited greater myogenic colony formation efficiency both in young (n=20) and old age (n=8), compared to age-matched WT controls (Figure 22a). Moreover, this

improved myogenic colony formation was reversed by treating IL-6 KO satellite cells with recombinant IL-6 in culture (n=5, Figure 22b).

To further test the myogenic function of IL-6 in satellite cells *in vivo*, IL-6 KO satellite cells were transplanted into pre-injured IL-6 KO or WT recipient muscles and evaluated for engraftment after 4 weeks. For this experiment, IL-6 KO mice were crossed to GFP transgenic mice, which express green fluorescent protein (GFP) under control of the ubiquitous  $\beta$ -actin promoter, in order to visualize engrafted myofibers by epifluorescence (Figure 22c,d). GFP<sup>+</sup> IL-6 KO satellite cells engrafted better than GFP<sup>+</sup> WT satellite cells when transplanted into IL-6 KO recipients as measured by the number of engrafted myofibers (n=5-8, Figure 22d,e). This effect was not observed in WT recipients, possibly because IL-6 KO satellite cells become exposed to IL-6 when transplanted into WT muscle. Interestingly, the average size of myofibers engrafted by IL-6 KO satellite cells tended to be greater than WT satellite cells regardless of the genotype of the recipient, suggesting that the growth of engrafted myofibers depends more on intrinsic, rather than extrinsic, expression of IL-6 (n=5-8, Figure 22f). It is also important to note that the higher engraftment efficiency of IL-6 KO satellite cells in IL-6 KO recipient could be due to ameliorated host inflammatory response in the complete absence of functional IL-6, which inflict less damage on the transplanted satellite cells than the WT environment, ultimately allowing for greater satellite cell survival and producing more engrafted myofibers. It has previously been demonstrated that myoblast transplantation into wild-type animals leads to cell death for 95-99% of cells within days of transplant (Tremblay, 1997). The authors of this study attributed acute myoblast death to an innate immune/inflammatory response mounted against transferred cells by the host.



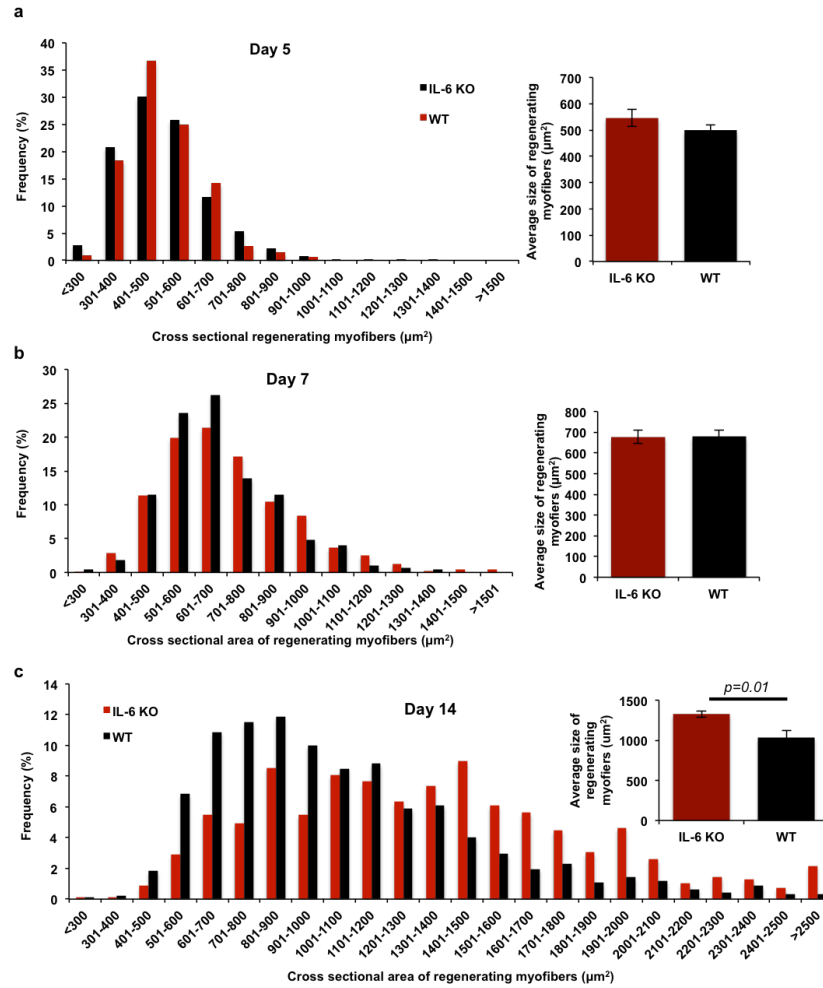


**Figure 22.** IL-6 restricts satellite cell function. (a) Myogenic colony formation efficiency of satellite cells isolated from young and aged IL-6 KO mice and WT mice. Knockout of IL-6 improved myogenic formation both at young and old age. (b) Treatment of IL-6 to IL-6 KO satellite cells in culture restored the myogenic colony formation level to that of WT satellite cells. (c) Experimental design of satellite cell transplantation. GFP<sup>+</sup> satellite cells or IL-6 KO; GFP<sup>+</sup> satellite cells were injected into recipient muscle injured with cardiotoxin 24 hours before transplantation. (d) Representative engraftment of GFP<sup>+</sup> satellite cells or IL-6 KO; GFP<sup>+</sup> satellite cells at 4 weeks after transplantation. (e) Quantification of the number of engrafted myofibers of GFP<sup>+</sup> satellite cells or IL-6 KO; GFP<sup>+</sup> satellite cells in IL-6 KO or WT recipient muscles. (f) Quantification of the average size of engrafted myofibers. Scale bar = 30  $\mu$ m. All data are presented as mean  $\pm$  SEM.

***IL-6 negatively affects muscle regeneration and strength in young mice.***

IL-6 KO mice were examined for their regenerative capacity. Young IL-6 KO mice showed greater cross sectional area of regenerating myofibers than young WT mice at day 14 after cryoinjury (n=5-8; Figure 23c). However, regenerating myofibers of IL-6 KO mice assessed at earlier time points after cryoinjury, day 5 and day 7, were not distinguishable from those of WT mice (n=5-8; Figure 23a,b). Also, the histology of muscle tissues in the resting state (without injury) was not different between IL-6 KO and WT mice (data not shown). This result points to potentially pleiotropic effects of IL-6 in muscle healing. That is, it is possible that damage-induced increases in IL-6 expression, which have been shown to be necessary at the onset of injury for successful myoblast proliferation and differentiation, may act later to inhibit myoblast fusion into multinucleated myofibers (Zhang et al., 2013). At earlier time points after injury (days 5 or 7), other inflammatory factors, such as TNF- $\alpha$  and members of the interleukin family, are likely able to compensate for the role of IL-6, resulting in similar level of muscle regeneration in IL-6 KO and WT mice at day 5 and 7 after injury. In the later stages of muscle regeneration, on the other hand, IL-6 might change its role, which might not be replaced by other inflammatory factors. Such a model would explain the different results at different time points after the dry ice injury experiment on IL-6 KO and WT mice.

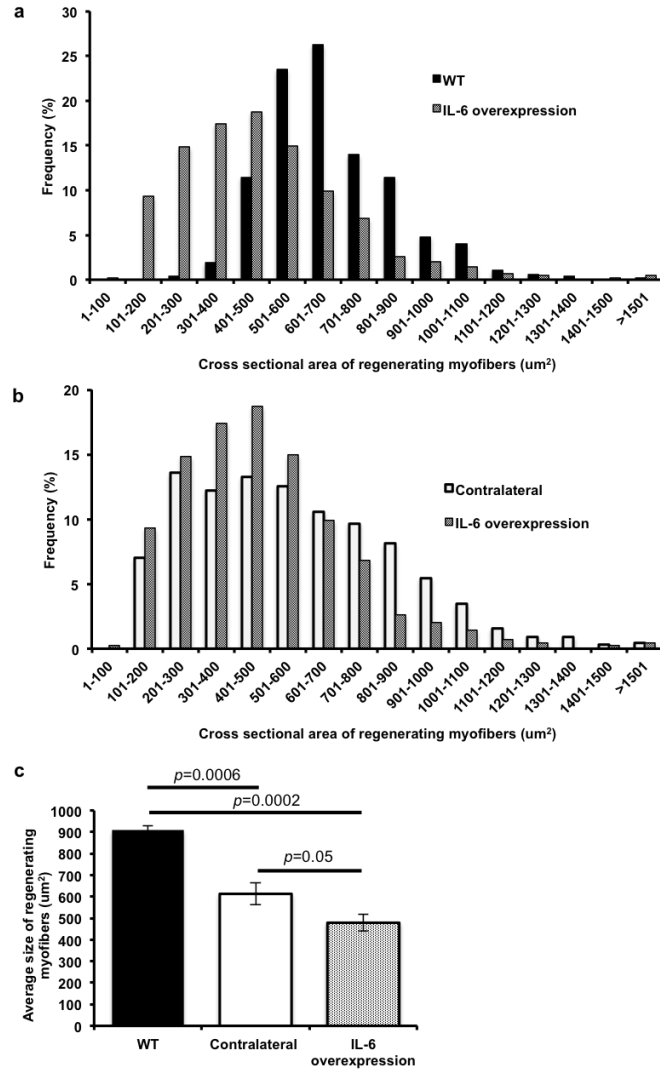
Next, young IL-6 KO and WT mice were subjected to prolonged IL-6 exposure in order to mimic the chronic inflammatory state of aged mice. Mice were injected with 300 pg/kg of human recombinant IL-6 (or PBS as a control) twice a day intraperitoneally for three weeks. The dosage of injection was determined to produce circulating IL-6 levels comparable to those of aged mice (Figure 21a) or other mouse models for metabolic diseases (Dal Kim, 2013). The amount of injected IL-6 was much lower than serum IL-6 levels observed in mice in acute inflammatory phase or with sepsis (Hua Huang, 2009; Casey, 1993). After three weeks of IL-6



**Figure 23.** Depletion of IL-6 improves muscle regeneration in young mice. Regeneration of skeletal muscle at day 5 (a), 7 (b), 14 (c) after cryoinjury in IL-6 KO and WT mice. IL-6 KO mice show improved muscle regeneration starting from day 14 after cryoinjury. All data are presented as mean  $\pm$  SEM.

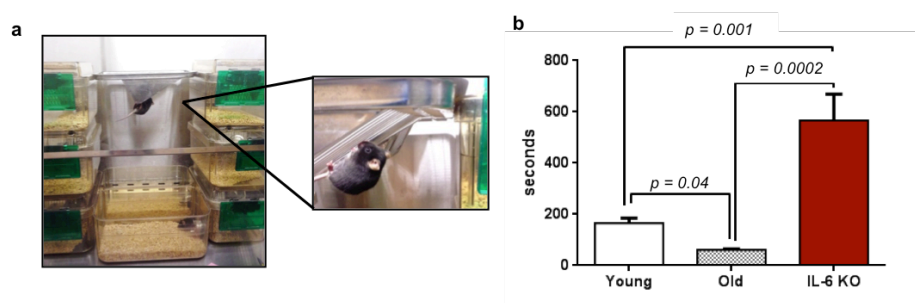
injection, mice were cryoinjured in the TA muscle and the muscles were harvested at day 14 after injury for analysis. However, daily injection of IL-6 did not cause any change in muscle regeneration in IL-6 KO or WT mice (n=3; Supplemental figure 9a,b). A trend of decreased colony formation efficiency of satellite cells isolated from IL-6 KO mice injected with IL-6 was observed, although it was not statistically significant (n=7; Supplemental figure 9c). One point of concern is that the half-life of IL-6 is less than 6 hours in serum when not bound to soluble IL-6 receptors. It is possible that injected IL-6 might be degraded much faster than expected, providing only a brief boost in serum IL-6. At the same time, the 3-week period of IL-6 exposure might not have been enough to mimic the muscle niche of aged mice.

This study was repeated using electroporation of an IL-6-overexpressing plasmid into the mouse TA muscle (as previously described by Baltgalvis et al. (2008)). Baltgalvis et al. showed that the electroporated quadriceps continuously secretes IL-6 into the bloodstream, increasing systemic levels of IL-6. This is therefore a more realistic way to simulate chronic IL-6 elevation in young mice. TA muscles were cryoinjured 3 weeks after electroporation of IL-6-expressing or empty vector plasmid (Manderson et al., 2007). mRNA levels of IL-6 in the muscles electroporated with IL-6 plasmid measured by qPCR at harvest showed 437 fold increases, as compared to IL-6 level in muscles with electroporation of empty vector (n=7,  $p=0.007$ ). Muscles with IL-6 overexpression showed drastic decrease in the size of regenerating myofibers starting at day 7 after injury compared to control muscles electroporated with empty vector under the same condition (n=6, Figure 24a). The contralateral of TA muscles with IL-6 plasmid electroporation also showed reduced muscle regeneration, possibly due increased circulation of IL-6 produced by the electroporated muscle (n=7, Figure 24b). Regenerating myofiber size was smallest in the IL-6 overexpressing muscles, suggesting that higher levels of IL-6 in the microenvironment causes worse muscle regeneration (Figure 24b).



**Figure 24.** Overexpression of IL-6 deteriorates muscle regeneration in young mice. IL-6 overexpression was induced by electroporation of IL-6-expressing plasmid. Young WT mice were cryoinjured at 21 days after IL-6 overexpression, and injured muscles were harvested at day 7 after injury. Overexpression of IL-6 caused poor muscle regeneration (a) and also in contralateral muscle (b). (c) Average size of regenerating myofibers also show decreased regenerating myofiber size with overexpression IL-6. All data are presented as mean  $\pm$  SEM.

Based upon these experiments, I conclude that IL-6 is restrictive for the regenerative ability of skeletal muscle. This effect of IL-6 is likely mediated through extracellular IL-6, potentially in combination with IL-6 expression in satellite cells, which increases with age and reduces the myogenic potential of satellite cells. In addition, IL-6 seems to have a negative impact on whole muscle strength and endurance, which was quantified by modifying an existing wire hang protocol obtained from the Wellstone Muscular Dystrophy Center (Washington, DC). Mice were allowed to hang upside-down from wire bars at a height of 35cm, and latency-to-fall was measured based on three timed attempts per trial (Figure 25a). Three trials in the period of a week were performed on each set of mice. Young IL-6 KO (n=6) mice exhibited significantly greater muscular endurance in the wire hang test compared to either young (n=8) or old (n=7) WT mice, as measured by the amount of time hung on (Figure 25b).

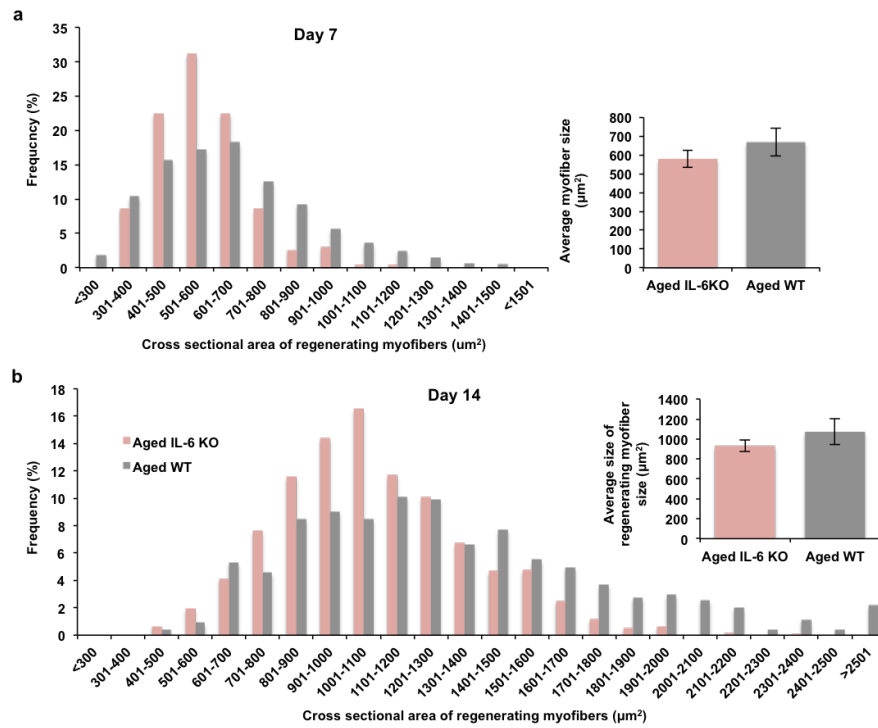


**Figure 25.** IL-6 KO mice show improved muscle strength and endurance. Latency to fall was measured in young and aged WT and young IL-6 KO mice hanging on mesh grid at 30 cm high. IL-6 KO mice significantly increased latency to fall as compared to age-matched WT or aged WT. All data are presented as mean  $\pm$  SEM.

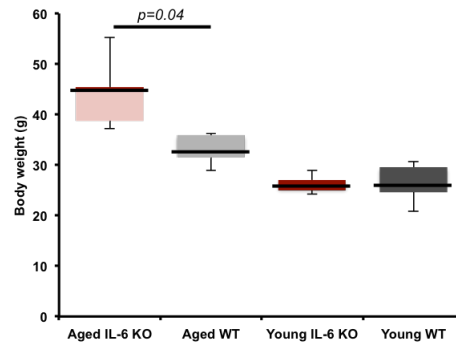
### ***Depletion of IL-6 is not sufficient to reverse muscle aging.***

Based on the results described above for young mice, I hypothesized that life-long depletion of functional IL-6 could be helpful for delaying muscle aging. To test this hypothesis aged IL-6 KO mice were examined for the myogenic potential of satellite cells and muscle regeneration. Colony formation efficiency of satellite cells was significantly higher in aged IL-6

KO mice than in aged WT mice, and almost comparable to young WT, as shown in Figure 22a. However, aged IL-6 KO mice failed to show preservation of muscle regeneration (Figure 26). Size distribution of regenerating myofibers in aged IL-6 KO mice showed a higher frequency of smaller myofibers after cryoinjury compared to littermate controls. Although this trend did not reach statistical significance, it implies that IL-6 is required, to some extent, for aged muscle to recover from injury. Though unexpected, the reduced regenerative ability of aged IL-6 KO mice, as compared to age-matched controls, may in fact be caused by the fact that IL-6 KO mice are notably obese when aged. In fact, it was reported that by 9 months of age IL-6 KO mice become obese and diabetic due to reduced energy expenditure (Wallenius, 2010). By 24 months of age, IL-6 KO mice weighed 37% more than age-matched WT mice (n=6), whereas young IL-6 KO and WT mice had no difference in weight (n=10, Figure 27). Since the obesity of mice gives rise to a slew of deleterious effects, ranging from liver inflammation to an impaired immune response, the beneficial effect of IL-6 depletion observed in young mice might not have been recapitulated in aged mice (Matthews, 2010). It has been shown that maturation of regenerating myofibers is suppressed in mice fed with high fat diet for 8 months (Hu, 2010). I also witnessed that 12 weeks of high fat diet treatment in mice beginning at 2-months of age is enough to impair muscle regeneration in young mice (n=4, Figure 28b,c). Interestingly, however, young IL-6 KO fed with high fat diet were protected from the obesity-associated decline in muscle regeneration (Figure 28a,c). Based on these data, I conclude that IL-6 plays a restrictive role in muscle regeneration by negatively affecting satellite cell survival and myofiber growth in young, relatively healthy mice. However, due to the multifactorial roles of IL-6 in many types of tissue, the accumulated effects global deletion of IL-6 results in obesity and associated metabolic complications over time, which ultimately disturb muscle maintenance through IL-6-independent pathways.

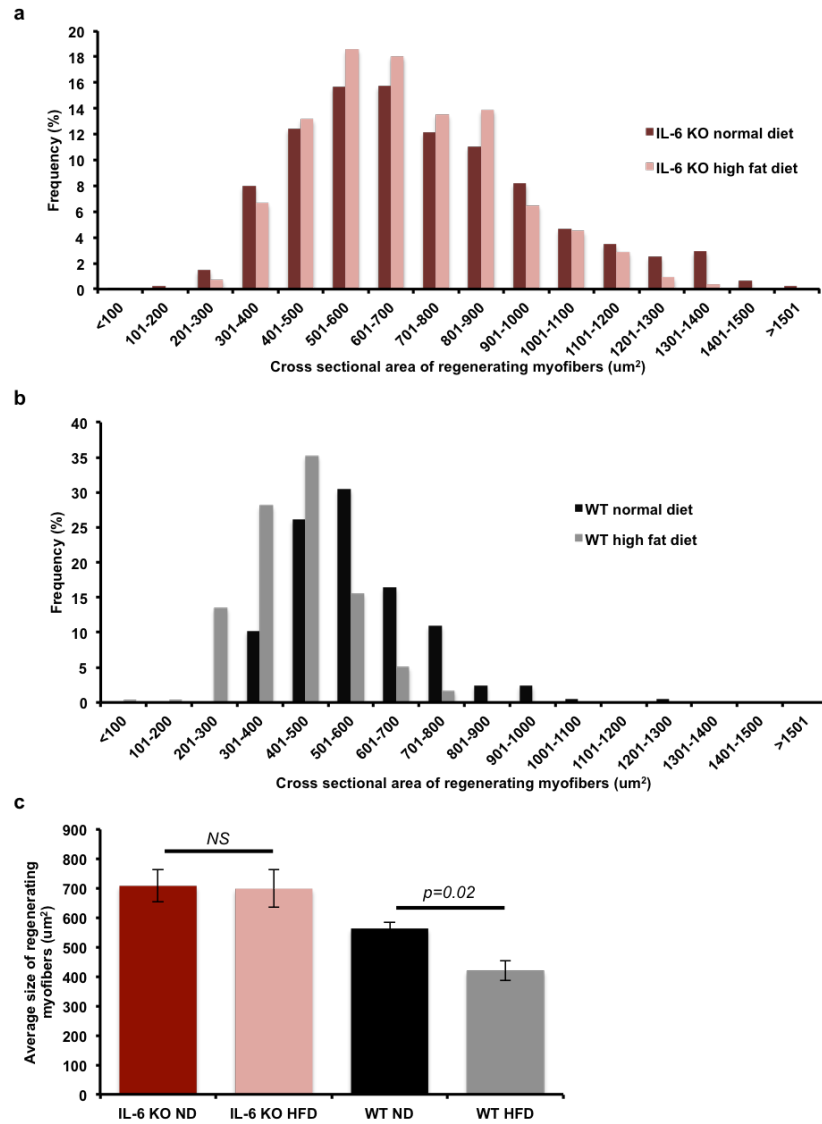


**Figure 26.** Depletion of IL-6 is not sufficient to improve muscle regeneration in aged mice. Aged IL-6 KO did not recapitulate the beneficial effect of IL-6 in muscle regeneration shown at day 14 after injury. IL-6 KO rather exhibited a slight trend of decreased regenerating myofiber size. All data are presented as mean  $\pm$  SEM.



**Figure 27.** Body weight of young and old IL-6 KO mice. Aged IL-6 KO mice have significantly increased body weight compared to age-matched WT, while young IL-6 KO and WT do not difference in body weight. All data are presented as mean  $\pm$  SEM.





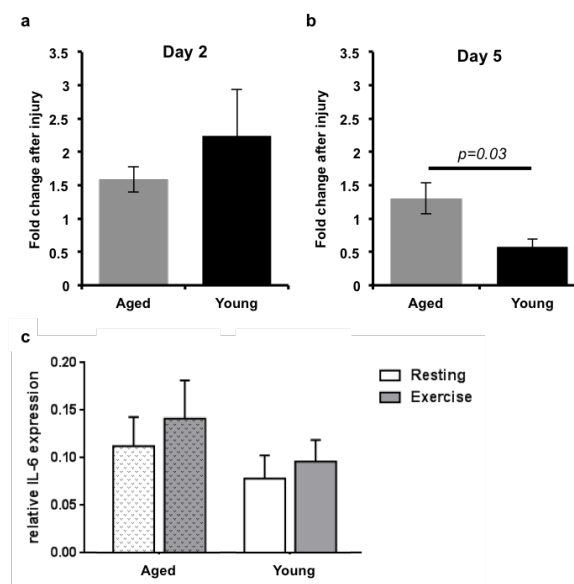
**Figure 28.** Young IL-6 KO mice are protected from high-fat diet induced decline in muscle regeneration. IL-6 KO mice and WT mice were treated with high fat diet or normal diet for 12 weeks and subjected for dry ice injury. High fat diet did not cause impaired muscle regeneration in IL-6 KO mice (a), unlike in WT mice, which showed significantly decreased regenerating myofiber size (c). All data are presented as mean  $\pm$  SEM.

***Regulation of IL-6 expression in muscle after injury is altered in aged mice.***

In parallel, I also asked if IL-6 is regulated differently in young and old mice during muscle repair. I examined changes in IL-6 levels in muscles of young and old WT mice after two forms of insult to muscle: cardiotoxin injury and acute exercise. It is well known that the immune response to injury involves inflammatory factors, produced by monocytes and macrophages that are recruited to sites of damage. For direct muscle injury, cardiotoxin was injected into the TA, 2 and 5 days prior to harvest. Both injured and uninjured TAs of young (n=6) and aged (n=4) mice were collected for qPCR analysis, and data are expressed as a ratio between injured and uninjured relative expression of IL-6, in order to see if the injury-induced IL-6 expression is altered with aging (Figure 29a,b). I found that at 2 days after cardiotoxin injury, young and aged mice had approximately 120% and 50% increase in IL-6 expression relative to uninjured controls respectively. At 5 days after injury, aged mice still showed increase in IL-6 expression about 30% whereas young mice showed decreased IL-6 expression relative to uninjured controls. It is notable that effective muscle injury repair requires a tightly controlled inflammatory response, with both inflammatory and anti-inflammatory cytokines necessary to produce the resulting beneficial effect. Proinflammatory monocytes and neutrophils infiltrate into injured areas and differentiate into M1-type macrophage shortly after damage to clear the damage and release a number of cytokines/chemokines, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and MCP-1 (Kharraz, 2013). In the later stages of regeneration, M2 polarization of macrophages reduces the levels of TNF- $\alpha$ , IL-6, and MCP-1, and promotes myoblast differentiation and fusion (Kharraz, 2013). It is therefore likely that the prolonged activation of IL-6 detected in aged, cardiotoxin-injured skeletal muscle might prevent transition to an adequate anti-inflammatory response after muscle injury.

To test inflammatory response after another form of muscle damage, young and old mice were subjected to exercise. Previous studies have found that contracting skeletal muscle responds to exercise by synthesizing and releasing IL-6 into the plasma (Fischer, 2006;

Febbraio, 2002b). Whole skeletal muscle was immediately harvested after one hour of uphill treadmill running and analyzed for expression of IL-6. Although the difference of means between whole muscle IL-6 transcription in resting and exercise states failed to reach statistical significance, both young and old mice tend to show a similar increase in average IL-6 expression after exercise (n=7, Figure 29c). Since not all mice reached exhaustion after one hour of running, it is possible that the rigorous activity in the exercise protocol was not adequate to cause proper inflammatory response. The duration of exercise or speed of the treadmill need to be increased to further study the regulation of IL-6 in young and aged mice during/after exercise.



**Figure 29.** Regulation of IL-6 expression in muscle after injury is altered in aged mice, but not after exercise. Change in gene expression of IL-6 in young and aged muscles after day 2 (a) and 5 (b) after cardiotoxin injury was compared by qRT-PCR. (c) IL-6 gene expression in young and aged muscles at resting state or after exercise was analyzed by qRT-PCR. All data are presented as mean  $\pm$  SEM.

## ***Discussion***

In summary, IL-6 has negative impact on muscle regeneration as shown by increased survival of IL-6 KO satellite cells in culture and in transplants, and greater myofiber growth after injury in young IL-6 KO mice. Satellite cells isolated from aged IL-6 KO mice showed further improvement in myogenic function of satellite cells in culture, but global deletion of IL-6 resulted ultimately in obesity and several associated metabolic diseases as mice age, causing declining muscle regenerative potential after injury as a secondary effect of obesity. Indeed, tocilizumab, the first IL-6 receptor-inhibiting monoclonal antibody approved recently for the treatment of rheumatoid arthritis and other inflammatory disorders, was reported to have side effects of weight gain and hyperlipidaemia, as reviewed by Febbraio et al. (Febbraio, 2010). This observation has been used to demonstrate the role of IL-6 in glucose homeostasis. Hence, while IL-6 is clearly pro-inflammatory in a range of diseases including sarcopenia, it is important to note that completely blocking IL-6 signalling can result in weight gain and hyperlipidaemia. The effect of hyperlipidemia on skeletal muscle mass and the interaction between adipose tissue stores and skeletal muscle size remain largely unknown. Despite the side effect of hyperlipidemia, the fact that transplanted IL-6 KO satellite cells show greater engraftment efficiency and greater myofiber size could suggest the possible utility of temporary inhibition of IL-6, perhaps through the use of IL-6-neutralizing antibodies, for increasing transplant efficiency in experimental, and perhaps clinical, applications.

Further study is required to understand how IL-6 affects the survival and/or proliferation of satellite cell in culture as shown in colony formation assay in figure 22a. It is possible that pro-apoptotic pathways or cell cycle regulation are altered in IL-6 KO satellite cells, resulting in higher cell survival and an increased colony formation rate in culture. Aged satellite cells were recently reported to switch to irreversible senescence from reversible quiescence (Sousa-Victor, 2014) and exhibit increased rate of apoptosis with up-regulation of cell death genes (Fulle,

2013), providing insight into the reduced myogenic colony forming function of aged satellite cell and the potential role of IL-6 in satellite cell function.

A recent study showed that IL-6-activated STAT3 signaling hinders satellite cell expansion during muscle repair by promoting myogenic lineage progression and asymmetric division of satellite cells, suggesting a causal role of IL-6 in the development of sarcopenia (Tierney, 2014). STAT signaling was also reported to be up-regulated in aged satellite cell, and genetic knockdown and pharmacological inhibition of Janus kinase (JAK) 2 and STAT3 expression in satellite cells stimulated symmetric cell division in culture and enhanced their ability to repopulate the satellite cell niche after transplantation into regenerating muscle (Price, 2014). In addition, the levels of STAT3 are elevated with muscle wasting as a downstream effector of IL-6 (Zhang, 2013), and STAT3 has been implicated in the regulation of self-renewal and stem cell fate in several tissues (Kiger, 2001). These results match in part with the trend of improved muscle regeneration in young IL-6 KO mice at day 14 after injury and greater engraftment efficiency of IL-6 KO satellite cell than WT satellite cells (Figure 22c,d,e, 23c). However, IL-6 KO and WT satellite cells used in this study did not show a significant difference in the level or timing of proliferation or differentiation in culture (Supplemental figure 10). It is possible that WT satellite cells isolated in culture do not produce sufficient amount of IL-6 to activate STAT3 signaling to the level to have an impact on the proliferation and differentiation of WT satellite cells. Assessing the levels of STAT3 signaling in IL-6 KO and WT satellite cells in culture will be helpful to explain how proliferation and differentiation of IL-6 KO satellite cell remain unaffected as compared to those of WT satellite cells.

Greater regenerating myofiber size in young IL-6 KO mice corroborates the catabolic role of IL-6 in muscle. As previously mentioned, IL-6 has been indicated as a potent stimulator of muscle proteolysis in many diseases and conditions associated with muscle atrophy. More interestingly, a recent paper reported induction of IL-6 and MuRF-1 by PGE2 in human skeletal

muscle (Standley, 2013), providing another interesting connection between muscle mass regulation and lipid metabolism that was briefly discussed in Chapter 3. Lastly, studying the regulation of proteolytic activity of IL-6 in satellite cells might help further understanding of the mechanism(s) underlying age-dependent functional decline of satellite cells.

## **Chapter 5.**

**Inhibition of NF- $\kappa$ B activity by up-regulation of HSP72 is not sufficient  
to reverse muscle aging**

## ***Preface***

Muscle wasting, or atrophy, describes a progressive loss of muscle mass that occurs as a consequence of denervation, injury, and joint immobilization (Tawa, 1994; Mitch, 1996). Frequently observed in patients of muscular dystrophies as well as cancer, AIDS and other systemic diseases including sarcopenia, muscle atrophy primarily results from accelerated protein degradation in affected muscle cells (Tawa, 1997). Protein degradation can be achieved in two ways—autophagy-lysosome pathway, or ubiquitin-proteasome pathway. Autophagy-lysosome pathway is usually non-selective and involves a number of proteases, whereas the ubiquitin-proteasome pathway selectively targets proteins for ubiquitination, which is recognized by ubiquitin receptors at the proteasomes (Dikic, 2009). Hyper-activation of the ubiquitin-proteasome pathway has been implicated in the degradation of intracellular skeletal muscle proteins (Tisdale, 2009). Interestingly, TNF- $\alpha$ -induced NF- $\kappa$ B activation increases ubiquitin-conjugating activity and up-regulates the ubiquitin-conjugating E2 enzyme, Ubch2 (Li, 2003), and inhibition of NF- $\kappa$ B protects against TNF- $\alpha$ -induced protein degradation in cultured muscle cells (Li, 2000). Expression of the E3 ligase MuRF1, a mediator of muscle atrophy, was increased in mice with muscle-specific activation of NF- $\kappa$ B activity (Cai, 2004), and blockade of the ATP-dependent ubiquitin-proteasome pathway with the inhibitor, MG132, inhibited NF- $\kappa$ B activity (Ortiz-Lazareno, 2008). Moreover, it is evident that IL-6 is involved in cancer cachexia, sepsis and acidosis (Bodell, 2009; Strassmann, 1992; Schaap, 2006), and induces skeletal muscle protein breakdown (Goodman, 1994). Based on results in previous chapters presenting the detrimental role of elevated NF- $\kappa$ B and IL-6 activity in muscle, I postulated that age-associated chronic inflammation and accelerated proteolysis in muscle tissue might be closely related.

Regulation of cellular processes responsible for protein quality control is known



collectively as protein homeostasis, or “proteostasis” (Bucciantini, 2002; Balch, 2008; Powers, 2009). A proper balance between synthesis, maturation, and degradation of cellular proteins is mandatory for maintaining proteostasis, which is essential for most cellular functions, including replication of genetic material, catalysis of metabolic reactions, maintenance of cellular architecture, signaling and immune responses (Balch, 2008; Powers., 2009). As regards stem cell function, proteostasis has been implicated as an important determinant for maintaining the stem cell identity and function of human embryonic stem cells and hematopoietic stem cells (HSCs) (Vilchez, 2012; Warr, 2013; Mortensen, 2011).

Proteostasis is regulated by a complex protein network that monitors the concentration, subcellular location and folding of proteins and includes molecular chaperones and folding enzymes, as well as pathways that drive protein degradation when needed, through the proteasome, lysosome and autophagy pathways (Balch, 2008; Powers, 2009; Taylor, 2011). Defects in proteostasis commonly lead to aberrant folding, toxic aggregation and accumulation of damaged proteins, which can in turn cause cellular damage and tissue dysfunction (Bucciantini, 2002). Indeed, age is one of the main risk factors for most diseases associated with protein misfolding, including neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, Huntington’s disease and amyotrophic lateral sclerosis (Moreno-Gonzalez, 2011). This association suggests that aged cells may be more prone to form and accumulate misfolded protein aggregates, which might lead to hyper-activation of proteolytic pathways (Taylor, 2011; Morimoto, 2009).

Recent studies have reported age-dependent deficits in the activities of both the autophagy-lysosomal and the ubiquitin-proteasome systems, the principal proteolytic systems implicated in protein quality control (Rubinsztein, 2011; Tomaru, 2012). Yet other studies have challenged this view, showing instead that autophagic potential or proteasome activity remains intact in aged cells, and attributing age-related disruptions in proteostasis mainly to increasing

damage caused by metabolic stress that overwhelms the protective capacity of proteolytic systems (Warr, 2013; Cook, 2009; Altun, 2009). In support of the latter view, it has been suggested that the age-related decline in proteostasis and specifically the inability to up-regulate chaperones in response to conformational stresses would trigger disease manifestation and, in turn, accelerate proteostasis collapse (Hartl, 2011). It has been known that substrate recognition and processing in the ubiquitin–proteasome system require a close cooperation with molecular chaperones. Notably, the overexpression of members of the heat shock protein (HSP) system has been shown to inhibit the formation of toxic oligomers and to prevent the formation of amyloid aggregates for different disease proteins (Hartl, 2011). More importantly, HSP70 overexpression was reported to prevent muscle atrophy by inhibiting the transcriptional activation of the E3 ligases, atrogin-1 and MuFR-1 (Senf, 2008). HSP70 overexpression also ameliorates dystrophic pathology in mice (Gehrig, 2012). On the basis of these findings, the pharmacological up-regulation of chaperone function promises to open new strategies for treating pathological states associated with aberrant protein folding and aggregation. However, a direct link between chaperone function and stem cell aging has not yet been demonstrated. To this end, I intended to study the link between chronic inflammation and proteostasis within skeletal muscle to determine whether compromised protein turnover is the mechanism by which chronic inflammation impairs muscle stem cell functionality and regenerative potential of aged muscle.

## **Results**

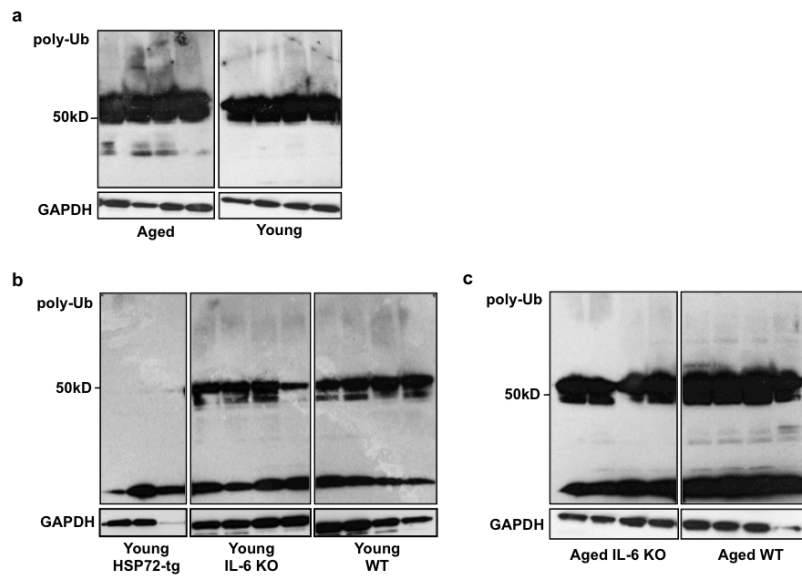
### ***Aged muscle accumulates ubiquitinated proteins, which can be removed by inhibition of inflammatory signals.***

First, I evaluated the amount of ubiquitinated proteins in young and aged muscles by western blot as a crude indicator of ubiquitin-proteasome-dependent protein degradation level.

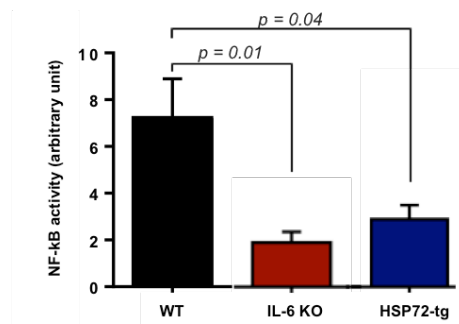
Ubiquitin antibody detected all ubiquitin conjugates in the protein extracts of young and aged muscle tissue, and showed greater amount of ubiquitinated proteins in aged mice (n=7; Figure 29a). This result also confirms previous studies in rat (Altun, 2010; Murata, 2011; Clavel, 2006). Moreover, overexpression of HSP72, an inducible isoform of HSP70, drastically reduced ubiquitinated protein levels in muscles (n=4, Figure 29b), which might imply that stabilization of protein folding by overexpression of chaperone protein reduces accumulation of misfolded proteins and thereby decreases the need of their clearance by the ubiquitin-proteasome pathway.

Ubiquitinated-protein level was also compared in muscles of young and aged IL-6 KO and age-matched WT mice in order to see if decreased systemic inflammation has any effect on ubiquitin-proteasome activity. IL-6 KO and WT mice did showed no difference in ubiquitinated-protein level at young age, but when aged, IL-6 KO mice accumulated less ubiquitinated-protein compared to aged WT (n=4, Figure 29b,c). Based on previous studies that pointed to IL-6 as a potent driver of proteolysis in muscle (Bodell, 2009; Strassmann, 1992; Schaap, 2006), it is possible that depletion of IL-6 diminished proteolytic activity in aged mice by preventing hyper-activation of the ubiquitin-proteasome pathway. The low inflammatory state of IL-6 KO mice was confirmed by decreased NF- $\kappa$ B activity in muscle measured by EMSA (n=5, Figure 30). In addition, HSP72-overexpressing mice showed inhibited transcriptional activity of NF- $\kappa$ B activity in muscle (Figure 30), as previously reported in a rat study (Senf, 2008). These data provide a line of evidence that low inflammatory state correlates with reduced level of ubiquitinated proteins during aging.

Accumulation of ubiquitin conjugates could be attributable to multiple factors—changes in the overall rate of protein ubiquitination in relation to the level of protein damage and misfolding, catalytic activity of the proteasome, or efficiency of deubiquitination. For complete understanding of the age-dependent regulation of ubiquitin-proteasome-dependent protein



**Figure 30.** Ubiquitinated protein level is increased in aged muscle. Level of total ubiquitin conjugate was measured by western blot against ubiquitin in protein extract of muscle tissues of young and aged WT (a), HSP72-tg and IL-6 KO mice (b), and aged IL-6 KO (c). GAPDH was used as a loading control.



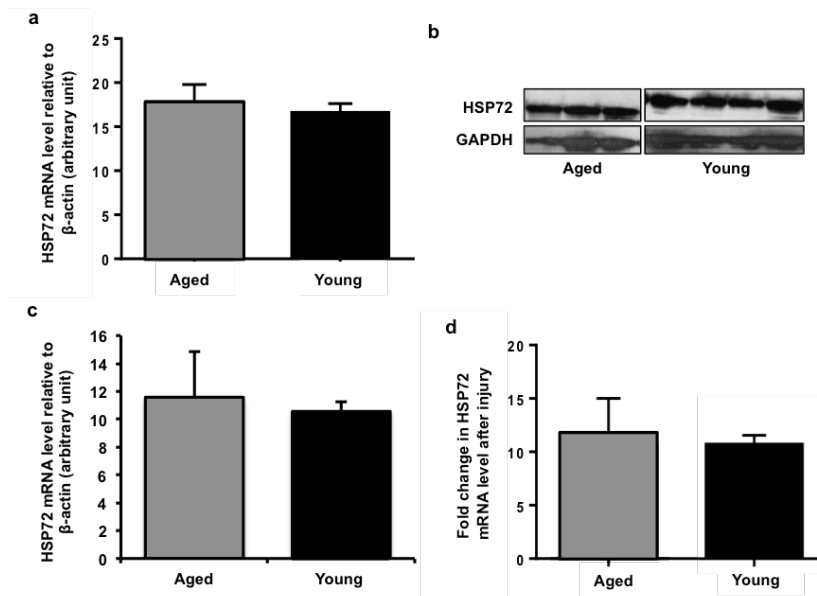
**Figure 31.** Decreased NF-κB in HSP72-tg mice and IL-6 KO mice. NF-κB activity was measured by EMSA in young WT, IL-6 KO, and HSP72-tg mice. All data are presented as mean  $\pm$  SEM.

degradation in muscle and possible role of HSP72 in the process, expression and activity level of proteasomes subunits, such as 20S and 19S, in these mouse models need to be analyzed.

***Regulation of HSP72 expression does not change with age.***

Next, I asked if chaperone proteins are regulated differently in young and aged mice. As previous studies have shown that inhibition of proteasome activity induces HSPs (Bush, 1997; Kim, 1999), I postulated that the expression level of HSPs in muscle could be indicative of proteasome activity. HSP72 mRNA and protein levels in muscle tissue were not different in young and aged mice (n=5, Figure 32a,b). Likewise, mRNA levels of HSP72 in satellite cells isolated from young and aged mice were similar (n=5, Figure 32c). In addition, since HSP72 expression is strongly up-regulated by cellular stress, induction of HSP72 mRNA level after cardiotoxin injury in young and aged muscle was also compared. Both young and aged mice showed a similar increase in HSP72 expression after injury, as shown in Figure 31d (n=6). Thus, injury-associated regulation of HSP72 does not appear to change with aging. Expression levels of other proteins in the HSP family (HSP90, HSP60, HSP27, HSF,  $\alpha$ B-crystallin) were also measured in young and aged muscle by qPCR and western blot, but no change was detected (data not shown).

Taken together, these data indicate that aged mice show increased levels of ubiquitinated proteins in muscle without changes in HSP72 expression. This result suggests that accumulation of ubiquitinated protein in aged muscle could reflect a failure to upregulate chaperone proteins in response to increasing demand from damaged and misfolded proteins. To test this possibility, I tested if increased expression of chaperones, particularly HSP70, in muscle is able to prevent muscle atrophy or reverse the age-related decline in muscle function by stabilizing proteostasis of muscle proteins.



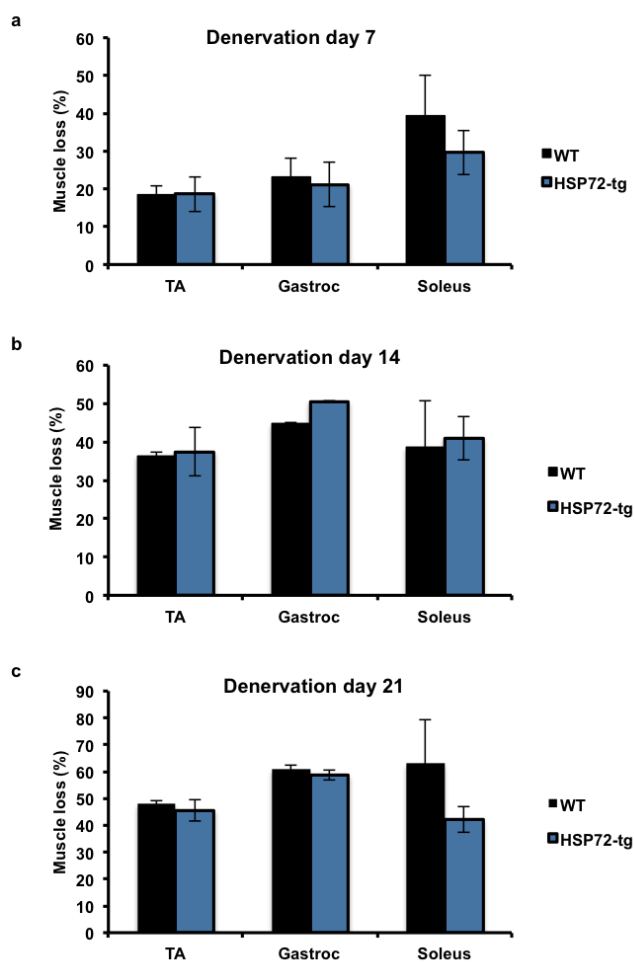
**Figure 32.** HSP72 level in young and aged mice. HSP72 expression level was measured by pRT-PCR and western blot. HSP72 expression in whole muscle tissues of young aged mice was not different at mRNA level (a) and protein level (b). (c) Expression of HSP72 gene in satellite cells of young and aged mice did not show difference. (d) Fold change in gene expression of HSP72 in muscle after cardiotoxin injury also did not show difference at different ages. All data are presented as mean  $\pm$  SEM.

### ***Overexpression of HSP72 does not affect regeneration or maintenance of skeletal muscle.***

A transgenic mouse model designed to globally overexpress HSP72 under the CMV promoter (HSP72-tg) was utilized to test if HSP72 has protective role in muscle. Up-regulation of HSP72 in the transgenic mice was validated by qPCR and western blot (Supplemental figure 11). HSP72-tg mice did not show a significant difference in muscle development or muscle mass at young age (Supplemental figure 12).

First, the potential role of HSP72 in muscle atrophy was studied by inducing atrophy through denervation in the hindlimb of HSP72-tg and WT mice. Motor innervation is known to be an important regulator of skeletal muscle mass and function, and denervation was reported to induce muscle atrophy through mechanisms involving the activation of FoxO and E3 ubiquitin

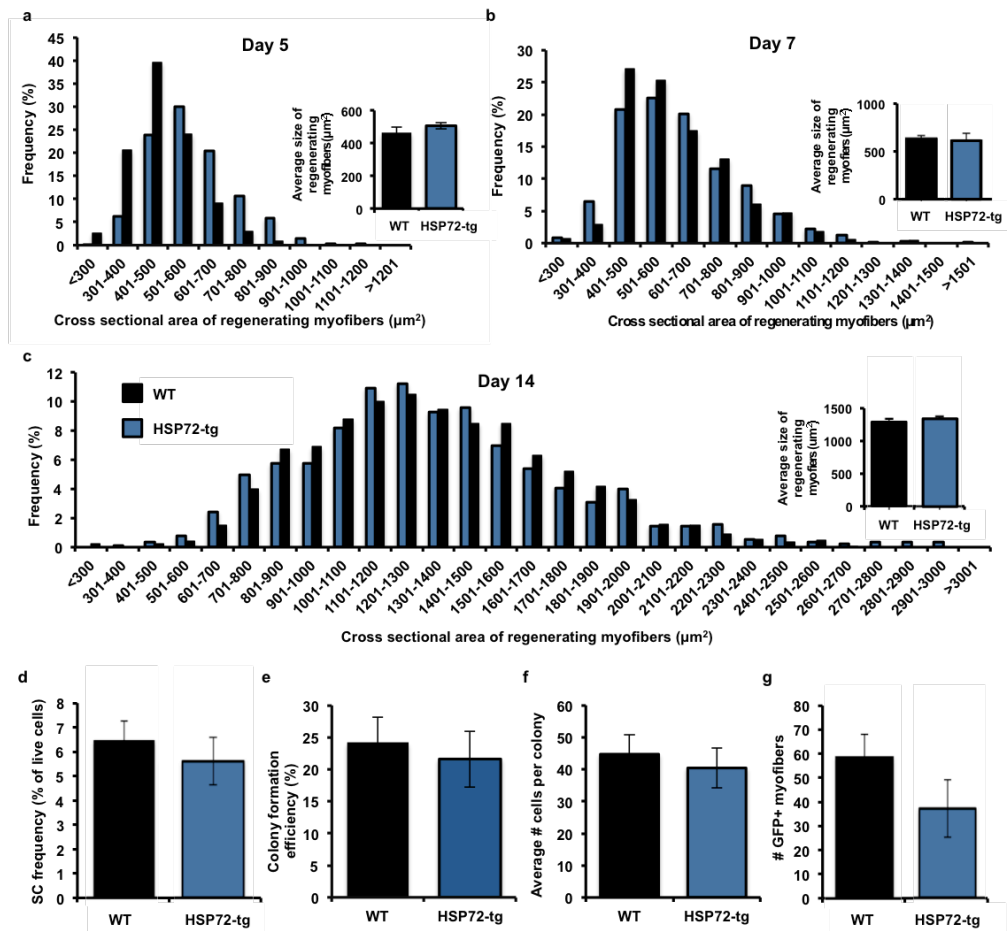
ligases (Tang, 2014). Animals were denervated by removing sciatic nerve, and changes in the mass of hindlimb muscles were measured 7, 14, and 21 days after denervation. Denervation caused significant loss of muscle mass in both HSP72-tg and WT mice, and the degree of muscle mass was not different between the two groups of mice at day 7, 14, or 21 after denervation (Figure 33a,b,c). Overexpression of HSP72 was not able to delay or hinder denervation-induced muscle atrophy.



**Figure 33.** HSP72 overexpression does not affect denervation-induced atrophy. Mass of TA, gastroc, and soleus muscle of denervated limb of HSP72-tg and WT mice was measured and compared to the contralateral controls without denervation, in order to calculate the percentage of muscle loss. Muscle loss was analyzed at day 7 (a), 14 (b), and 21 (c) after denervation. All data are presented as mean  $\pm$  SEM.

Muscle regeneration after cyoinjury and satellite cell function of young HSP72-tg mice was also evaluated (Figure 34). HSP72-tg mice exhibited a higher frequency of regenerating myofibers of greater cross-sectional size as compared to WT littermates at earlier time point after injury (day 5), possibly due to facilitated protein recovery after muscle damage (n=6, Figure 34a). However, this difference disappeared as muscle regeneration progressed (n=7,4; Figure 34b,c). Satellite cells of HSP72-tg mice were not distinguishable from satellite cells of WT mice in terms of satellite cell frequency, colony formation efficiency, or in vitro proliferation (n=8; Figure 33d,e,f). Also, GFP<sup>+</sup> satellite cells transplanted into HSP72-tg mice showed indistinguishable numbers of engrafted myofibers as compared to WT controls (n=4, Figure 33g). Studies of *Hsp70*<sup>-/-</sup> mice reported smaller myofiber size in resting muscle and severely impaired muscle regeneration after injury due to delayed inflammatory response, but no significant compromise in markers of satellite cell activation and proliferation in injured muscles (Senf, 2013). Potential role of HSP72 in recruitment of inflammatory cells in muscle after injury could explain the temporal improvement in muscle regeneration in HSP72-tg mice at day 5 after injury, when proper pro- and anti-inflammatory response is essential. On the other hand, HSP70 does not appear to play a critical role in myogenic progression of satellite cells. Interestingly, HSP72-tg mice showed significantly increased latency to fall in wire-hang test, indicating overall muscle strength and function is improved with HSP72 overexpression (Supplemental figure 13). Since the outcome of hanging performance of mice is through coordination of multiple aspects of muscle function, further study is necessary to understand the advantageous role of HSP72 in this context.

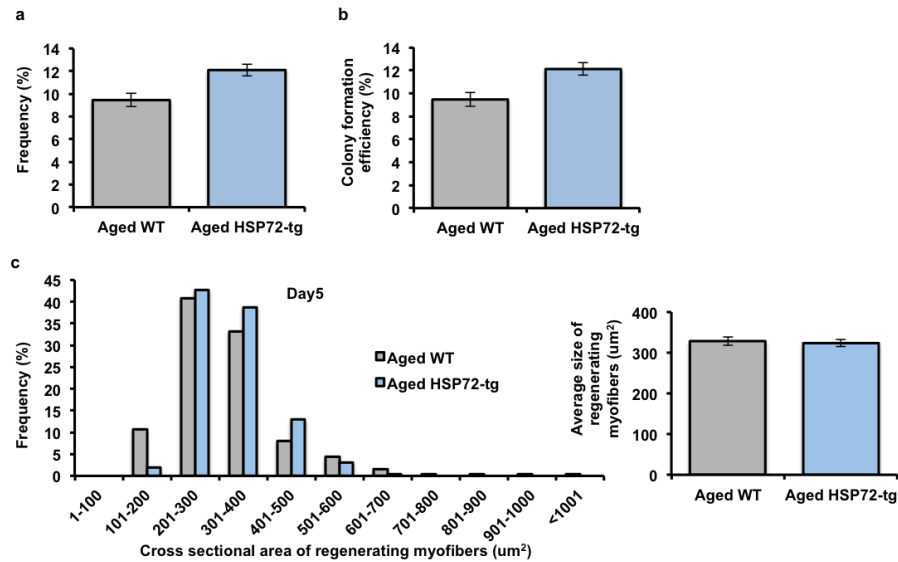




**Figure 34.** Satellite cell function and muscle regeneration does not change in young HSP72-tg mice. Muscle regeneration at day 5 (a), 7 (b), and 14 (c) after dry ice injury was compared in age-matched HSP72-tg and WT mice. Size distribution and the average size of regenerating myofibers did not show difference in HSP72-tg and WT mice. (d) Satellite cell frequency, (e) colony formation frequency, (f) average number of cells per myogenic colony, (g), and the level of engraftment of GFP+ satellite cells were also not different between HSP72-tg and WT mice. All data are presented as mean  $\pm$  SEM.

***Life-long overexpression of HSP72 does not protect muscle from age-related dysfunctions.***

Beneficial effects of HSP72 overexpression might not stand out at young age, because young muscles are not exposed as much to proteotoxic insults as aged muscles. For this reason, HSP72-tg mice were aged for 24 month in order to examine if HSP72 helps preventing sarcopenic symptoms. In aged mice, however, life-long overexpression of HSP72 was not sufficient to enhance the regenerative capacity of aged muscle as shown by regeneration of myofibers after cryoinjury (Figure 34c). The frequency and colony formation efficiency of satellite cells exhibited a slight trend of improvement, but the difference did not reach the level of statistical significance (Figure 35a,b). Although unexpected, it is possible that the role of HSP72 in recruiting inflammatory cells to damage site is no longer beneficial for muscle regeneration when muscle is chronically inflamed, as in aged mice.



**Figure 35.** Satellite cell function and muscle regeneration does not change in aged HSP72-tg mice. HSP72-tg mice were aged for 2 years and subjected for dry ice injury and satellite cell isolation. Satellite cells isolated from aged HSP72-tg mice were not different from aged WT mice in terms of frequency (a) and colony formation frequency (b). (c) Regeneration from dry ice injury was not improved in muscle of aged HSP72-tg mice. All data are presented as mean  $\pm$  SEM.

## ***Discussion***

Thus far, I have studied the potential role of HSP72 as a protective molecule combatting NF- $\kappa$ B-mediated protein degradation in aged muscle. In fact, increased ubiquitin conjugate level was detected in aged muscle, suggesting activated ubiquitin-proteasome proteolytic pathway. A previous study suggested that as aged muscle adapts to increased protein damage and misfolding, ubiquitin-proteasome proteolytic pathway becomes hyper-activated, eventually leading to collapse in proteostasis (Altun, 2010).

The associations between molecular chaperones and the ubiquitin–proteasome system represent a critical step in the response to proteotoxic damage. Whether attempts should be made to refold damaged proteins or degrade them instead requires a consideration of cellular economy. Defects in the quality-control mechanisms may have enormous consequences even if only slight imbalances occur between protein folding and degradation, as these imbalances can cause accumulated toxicity over time. Modulation of this system may provide a unique therapeutic target for degenerative diseases and pathologies associated with aging. This study has focused on the role of HSP70, as it is known to regulate NF- $\kappa$ B activity and is relatively well studied in muscle tissue, but the involvement of other HSP families in muscle aging, maybe in collaboration with HSP70, would also be interesting to study.

Regulation of HSP72 expression was shown to remain unchanged with age. Considering the basic role of HSP72 as a chaperone assisting protein folding, I tested if up-regulation of HSP72 helps to maintain muscle tissue. Global overexpression of HSP72 reduced NF- $\kappa$ B activity in muscle, but it was not sufficient to prevent muscle atrophy induced by denervation. In the case of disuse atrophy, however, overexpression of HSP70 prevented muscle loss by inhibiting NF- $\kappa$ B and Foxo3a activity (Senf, 2008). It is conceivable that the mechanism of muscle atrophy is different depending on how it is induced. Preventive role of HSP72 in age-related muscle atrophy is to be studied.

Muscle regeneration at early stages appeared to be improved in young HSP72-tg mice, possibly due to facilitated immune response as previously studied (Senf, 2013), but such an effect was not observed in aged HSP72-tg mice. As inadequate and excessive inflammatory response is often attributed to delayed muscle regeneration in aged mice, increased recruitment of inflammatory cells by up-regulated HSP72 might have adverse effects on regeneration of aged muscle.

Satellite cell function was not affected by HSP72 expression at any age examined here. A recent study in the hematopoietic system reported an important role of the autophagy-lysosome pathway in maintaining HSC pool in old mice by protecting HSCs from metabolic stress. However, satellite cells of young and aged mice in this study did not show any difference in mRNA expression of essential genes involved in autophagy (ULK1, ATG5, ATG7, FOXO3a) (data not shown). As quiescent satellite cells directly isolated from mice show low metabolic activity, further analysis on autophagic activity of proliferating or differentiating satellite cells should be done. It will be important to characterize the mechanisms that regulate proteostasis in muscle stem cells to determine if they differ from those in other cells and to assess how they may influence changes in stem cell function during aging.

## **Chapter 6.**

### **Conclusions and discussion:**

#### **Prevention of chronic inflammation for healthy aging**

Sarcopenia, the age-related loss of muscle mass, is a direct cause of an age-related decrease in muscle function that has an immediate impact on the quality of living in the old age. Decline in the regenerative capacity of muscle due to a decrease in functional satellite cell pool has been considered as one of reasons causing age-related muscle wasting. Based on previous studies showing the impact of aged systemic milieu on regenerative potential of muscle and other tissue functions (Conboy, 2003; Conboy, 2005b; Vilela, 2011; Loffredo, 2013; Elabd, 2014; Sinha, 2014; Katsimpardi, 2014), this study focused on the role of chronic inflammation in aging of satellite cell pool. Unproductive, low-grade chronic inflammation, characterized by up-regulation of the inflammatory responses due to the chronic antigenic stress that impinges throughout life upon innate immunity, has been implicated in the onset of many age-associated chronic diseases, including sarcopenia. In this notion, this study aimed to discover the molecular mediators of chronic inflammation and their mechanisms of action in the age-related decline in regenerative capacity of muscle. As a result, this study showed age-dependent up-regulation of NF- $\kappa$ B activity in muscle tissue, and discriminated the impact of NF- $\kappa$ B elevation/inhibition in skeletal muscle fibers versus satellite cells, and revealed a non-autonomous regulation of satellite cell regenerative function by NF- $\kappa$ B driven gene expression in regenerating myofibers. This result corroborates the importance of muscle stem cell niche, or microenvironment, in regulation of satellite cell function. It was also demonstrated that systemic inhibition of NF- $\kappa$ B activity promotes more robust muscle regeneration in aged animals, suggesting salicylate treatment as a potential therapeutics for sarcopenia.

In order to further dissect the molecular mechanism of non cell-autonomous effect of NF- $\kappa$ B in muscle, gene expression pattern in whole muscle of young and aged WT, and aged MISR mice was compared. Candidate genes that are differentially expressed between young and

aged WT muscle and also between aged WT and aged MISR muscle were validated and tested for their potential role in muscle regeneration. As a result, group V phospholipase A2, PLA2G5, was identified as a molecular mediator of age-dependent elevation of NF- $\kappa$ B activity, extending understanding of the pathophysiology of age-related loss of muscle regenerative capacity, and, at the same time, opening a new insight into the cross-regulation of lipid metabolism and muscle maintenance. Further study is required to clarify how PLA2G5 incurs inflammatory tone in muscle. Prostaglandins, enzymatically driven from fatty acids by phospholipase A2, have been known to regulate inflammation; and COX-2, an enzyme responsible for intermediate steps in sequential oxidation of arachidonic acid to produce prostaglandins, is the drug target of currently available non-steroidal anti-inflammatory drugs. Especially prostaglandin E2 (PGE2) has been implicated in muscle wasting which suggests PGE2 as an intriguing candidate to study for its potential role in satellite cell function (Standley, 2013).

In addition, this study revealed a restrictive role of IL-6, one of target genes of NF- $\kappa$ B and a pro-inflammatory cytokine widely observed as a biomarker of chronic inflammatory state of aged individuals, in the myogenic function of satellite cells. Global depletion of IL-6 improved satellite cell function and muscle regeneration in young mice; however, in aged mice, life-long depletion of IL-6 was not able to rescue impaired muscle regeneration even though satellite cell function was still enhanced. I conclude that this distinct effect of IL-6 depletion in young and old age is due to mature-onset obesity and obesity-induced diabetes in aged mice with IL-6 depletion. This result suggests an unexpected role of IL-6 in energy metabolism. Previously Wallenius et al. reported the anti-obesity effect of IL-6 is mainly exerted at the level of the central nervous system, possibly in the hypothalamus (Wellenius, 2002). It is possible that IL-6 has different effects on different tissues, and for this reason, it needs to be considered that there may be a small therapeutic window between the doses needed for beneficial effects and those associated with adverse effects and illness.

In parallel, HSP72 was studied as a part of the molecular pathway for inhibition of chronic inflammation or for mitigation of the damaging effects of chronic inflammation in muscle. Overexpression of HSP72 appeared to decrease protein degradation activity along with reduced NF- $\kappa$ B activity; however, it was not sufficient to improve the regenerative function of aged muscle or aged satellite cell. Based on previous reports about the beneficial effect of HSP72 in prevention of disuse atrophy (Senf, 2008), it is conceivable that HSP72 has a cytoprotective role in situations when muscle cells are undergoing excessive, unnecessary protein degradation. However, during muscle satellite cell proliferation/differentiation and myofiber growth when protein synthesis is expected to exceed protein degradation, the protective role of HSP72 might not be as prominent. In fact, treatment with BGP-15, a pharmacological inducer of Hsp72 currently in clinical trials for diabetes, improved muscle architecture, strength and contractile function in severely affected muscles of mdx dystrophic mice, by protecting fragile myofibers susceptible to an influx of  $\text{Ca}^{2+}$  (Gehrig, 2012). Although overexpression of HSP72 does not seem to be advantageous for satellite cell function and muscle regeneration, other aspects required for proper function of muscle, such as intact neuromuscular junction and energy metabolism, in HSP72-overexpressing mice might reveal additional role(s) of HSP in muscle. Also, regulation of protein homeostasis by other molecules or pathways in satellite cells, both quiescent and differentiating, in young or old mice, needs to be studied in order to understand the potential role of protein quality control in maintenance of functional satellite cell pool.

Thus far, I discussed about the impact of chronic inflammation and its molecular mediators and inhibitors on the regenerative capacity of aged muscle. The ultimate goal of uncovering the molecular basis of age-related chronic inflammation would be to develop a therapeutic strategy for improvement of muscle health and prevent muscle degeneration in patients or older individuals. A number of currently available regimens are suggested to promote healthy function during aging and to counteract chronic inflammation. Caloric restriction, for



example, decreases accumulation of metabolic byproducts such as reactive oxygen species, which have the ability to activate cell signaling cascades that include I $\kappa$ B kinase and MAPKs and initiate pro-inflammatory responses (Kim, 2000). Thus, caloric restriction delays the biological rate of aging and extends lifespan in a variety of organisms from yeast to primates, and protect against age-associated diseases including chronic inflammatory disorders such as cardiovascular disease and diabetes (González, 2012). A previous study from the Wagers lab showed increases the frequency and function of skeletal muscle stem cells in both young and old mice after short-term calorie restriction, potentially by increasing mitochondrial content and promoting oxidative metabolism (Cerletti, 2012).

Physical exercise is also well recognized as an important strategy for reducing the risk of chronic diseases, and many studies have been done to investigate its role in the improvement of the inflammatory profile (Beavers, 2010). Large population-based cohort studies consistently show an inverse association between frequency of physical activity and markers of systemic inflammation such as C-reactive protein (CRP), IL-6, and fibrinogen, in old age (Taffe, 2000; Reuben, 2003; Elosua, 2005). Although specific molecular mechanisms underlying the beneficial effects of exercise is still under active investigation, physical exercise is a promising strategy for reducing chronic inflammation. Also, changes in systemic inflammatory tone caused by physical activity stress an additional function of skeletal muscle as a secretory organ. As the largest organ in the body, skeletal muscle is capable of largely impacting the systemic milieu and communicating with other organs such as adipose tissue, liver, pancreas, bones, and brain, by its secretory phenotype. In this view, elevated NF- $\kappa$ B activity in aged muscle tissue would have global influence, in addition to the detrimental impact on local environment, contributing to systemic inflammation in aging animals.

These anti-inflammatory regimens are clearly capable of improving the overall health status of subjects, but development of therapeutic treatment for muscle wasting is also crucial

for sarcopenic/atrophic individuals whose physical and nutritional status is not suitable for restriction of calorie intake or frequent physical activity. For this purpose, elucidating the molecular profile, especially the secretory profile in skeletal muscle, underlying these anti-inflammatory regimens will be useful for mimicking the beneficial effects. I believe that NF- $\kappa$ B signaling pathway and its downstream and upstream effectors in muscle investigated in this study will not only provide potential targets for improving skeletal muscle regeneration but also further help to extend the healthspan of older adults and patients.

**Chapter 8.**  
**Materials and methods**

## ***Animals***

Young (2–3 month) and aged (24–26 month) C57BL/6 mice were obtained from pathogen-free breeding colonies at Jackson Laboratories and Charles River Labs. MISR (obtained from Dr. Steven Shoelson; Cai, 2004), Pax7Cre-ER mice (Nishijo, 2009), IL-6 KO (C57BL/6;129S2-Il6tm1Kopf/J; Kppf, 1994) mice, and GFP transgenic mice (C57BL/Ka- $\beta$ -actin-EGFP; Sherwood, 2004; Cerletti, 2008; Cerletti, 2012) were bred in house; flox-STOP-flox IKK mice (C57BL/6-Gt(ROSA)26Sortm1(lkbb)Rsky/J) were purchased from Jackson Labs. HSP72 over-expressing mice (initially obtained from Dr. Mark Febbraio) were successfully back-crossed for at least nine generations to C57BL/6 mice.

Fidelity of the Pax7-CreER line for satellite cell-specific gene expression has been validated previously (Nishijo, 2009). Tamoxifen (0.1 mg / g body weight) or corn oil vehicle (10  $\mu$ l /g body weight) was injected for 5 consecutive days after two weeks of sodium salicylate treatment. For sodium salicylate treatment, a feed containing 3 grams of sodium salicylate (Sigma Aldrich) per kg was created by Research Diets, and purchased along with control feed with the same nutritional content but lacking salicylate. Mice were provided sodium salicylate or control feed ad libitum for 6-8 weeks prior to experimentation. For IL-6 Injection, mice were injected with 300 pg/kg human recombinant IL-6 (Peprotech). IL-6 was dissolved in PBS to 16ng/ml, and a volume of 25 $\mu$ l was injected daily. Control mice were injected with PBS.

Animals were housed at the Biomedical Research Institute at Harvard University and animal facility at the Joslin Diabetes Center. All mice were housed and treated under approved protocols by relevant Institutional Animal Care and Use Committees.

## ***Satellite cell Isolation***

Single myofibers and myofiber-associated cells were prepared from intact limb muscles (extensor digitorum longus, gastrocnemius, quadriceps, soleus, tibialis anterior, and triceps

brachii), as previously described (Cerletti, 2008; Cerletti, 2012; Sinha, 2014). After isolation, all myofiber-associated cells were incubated in Hank's Buffered Salt Solution (Gibco) containing 2% donor bovine calf serum on ice for 20 min with the following antibodies: 30-F11 (1:200, anti-mouse CD45, phycoerythrin (PE) or allophycocyanin (APC) conjugate (eBioscience, San Diego, CA)); M1/70 (1:200, anti-mouse CD11b, PE conjugate, (eBioscience)); or 1:800, anti-mouse CD11b, APC conjugate, (eBioscience)); D7 (1:800, anti-Sca-1, Ly-6A/E, APC conjugate (eBioscience)),  $\beta$ 1-integrin (1:200, anti-mouse CD29, purified, (BD Pharmingen, San Jose, CA; or 1:400, anti-mouse/rat CD29, PE conjugate (Biolegend, San Diego, CA); CXCR4 (1:100, biotinylated anti-mouse CD184 (BD Pharmingen)), Streptavidin (1:100, Cy7-PE conjugate (eBioscience)), anti-armerian hamster IgG, fluorescein isothiocyanate (FITC) conjugate (1:100, eBioscience). Muscle stem cells, identified as CD45<sup>-</sup>Sca-1<sup>-</sup>Mac-1<sup>-</sup>CXCR4<sup>+</sup> $\beta$ 1-integrin<sup>+</sup> were sorted by Fluorescence Activated Cell Sorting (FACS) (Cerletti, 2008; Cerletti 2012). Previous studies have shown that >95% of CD45<sup>-</sup>Sca-1<sup>-</sup>Mac-1<sup>-</sup>CXCR4<sup>+</sup> $\beta$ 1-integrin<sup>+</sup> cells express Pax-7 (Cerletti, 2008). Live cells were identified as calcein blue positive (1:1000, Invitrogen, Carlsbad, CA) and propidium iodide negative (PI, 1mg/mL). Satellite cells were double-sorted to maximize purity (Cerletti, 2012).

Flow cytometry and cell sorting were performed at the Joslin Diabetes Center or HSCRB Flow Cytometry Cores. Sorting and analysis were carefully optimized for antibody titration and to achieve maximal cell purity and viability.

### ***Myogenesis assays***

For evaluation of myogenic function of satellite cells, colony formation assay and differentiation assay was performed. Formation of myogenic colony was used as a measurement of stem cell functionality of satellite cells to survive, self-renew and proliferate in culture. A single CD45<sup>-</sup>Sca-1<sup>-</sup>Mac-1<sup>-</sup>CXCR4<sup>+</sup> $\beta$ 1-integrin<sup>+</sup> satellite cell was seeded in each well

of collagen/laminin-coated 96-well plate. In order to coat the plates, wells were exposed to PBS containing 1µg/ml of collagen (Sigma) and 10µg/ml of laminin (Invitrogen) for at least one hour and then were briefly air-dried before addition of cell culture growth medium (F10, 20% horse serum, 1% Glutamax and 1% pen/strep) and 5ng/ml of bFGF (Sigma, St. Louis, MO). Afterwards, cells were cultured for 5 days at 37°C, with daily supplement of bFGF. Colony formation efficiency was analyzed using bright field microscopy on day 5 of culture as the percent of seeded wells that contained cell colonies.

For differentiation assays, satellite cells were sorted into coated 96-well plates at 3000 cells per well. Cells were cultured in growth media described above supplemented with bFGF for 5 days. At 5 days after plating, media were changed to differentiation media (DMEM, 1% GlutaMax, 1% pen-strep, and 2% horse serum) and cultured for an additional 2-3 days to allow proliferated satellite cells and myoblasts to differentiate and fuse to form myotubes. Myotubes were stained with anti-fast myosin (Sigma), and DAPI for myonucleus. Images were acquired using a Zeiss Observer D1 inverted microscope.

### ***Quantitative RT-PCR***

For RNA extraction, CD45<sup>-</sup>Sca-1<sup>-</sup>Mac-1<sup>-</sup>CXCR4<sup>+</sup>β1-integrin<sup>+</sup> satellite cells were deposited in Trizol (Invitrogen) and muscle tissues were homogenized in Trizol by Gentle MACS Dissociator (Miltenyi Biotech). cDNA was prepared using Superscript III Reverse Transcriptase Supermix Kit (Invitrogen). Real-time quantitative PCR was performed in an ABI 7900 machine (Applied Biosystems), using SYBR Green PCR mix (Quiagen). β-actin and hprt was used as a housekeeping gene, and gene expression levels were normalized to β-actin expression. Primers sequences are provided in Appendix (Table 2).

### ***Electrophoretic mobility shift assay (EMSA)***

All nuclear extraction procedures were performed on ice with ice-cold reagents. Nuclear protein was extracted from muscle tissue homogenized by glass Dounce homogenizers and Nuclear Extraction Kit from Panomics. Protein concentration of nuclear extract was measured using the BioRad DC Protein Assay (BioRad). Nuclear extracts were prepared for gel shift experiments by preincubating protein with poly(dI-dC) and either a biotin-labeled double-stranded oligonucleotide having consensus recognition sequences for NF- $\kappa$ B (5'-AGTTGAGGGGACTTTCCCAGG-3') and Oct-1 (5'-GTCGAATGCAAATCACTAGAA-3') (Panomics). Unlabeled probe was used as negative control, and nuclear extract prepared from HeLa cell line with stimulation of NF- $\kappa$ B activity was used for positive control (Panomics). Protein-DNA complexes were allowed to form for 30 min at 15°C, and the complexes were run on a native 5% TBE polyacrylamide gel (Criterion) and then transferred onto a Biodyne B nylon membrane (Pall). Oligos were fixed to the membrane by heat (80°C) for 1hr and then blocked and prepared for detection by streptavidin-horseradish peroxidase (HRP) solution according to the Panomics EMSA Detection Kit. Finally, membranes were exposed using enhanced chemiluminescence (ECL) Plus (Amersham). Results were visualized by autoradiography and quantified using Image J densitometry.

### ***Tissue injury and quantification of regenerating myofibers***

For cardiotoxin Injury, mice were anesthetized by isoflurane inhalation and a dose of 25 $\mu$ l of 0.03 mg/ml *Naja mossambica mossambica* cardiotoxin (Sigma) was injected directly into the tibialis anterior (TA) muscle using an insulin syringe. For cryoinjury, mice were anesthetized and a small piece of dry ice with flat surface was applied directly to exposed TA muscle for 5 s. The skin incision was closed with suture immediately after injury. This procedure generates a reproducible “wedge” injury in the muscle with a discrete border between uninjured and injured

muscle, and this border remains clear and distinct during regeneration of the injured tissue. Injured muscles were allowed to recover for 5, 7, 14 days before harvest. For quantification of muscle regeneration after injury, harvested muscles were fixed in 4% paraformaldehyde for 90 minutes and washed in PBS for paraffin sections, or frozen for frozen sections (10µm). Sections were stained with hematoxylin and eosin (H&E), and a series of pictures panning across the regenerating area in the cross-section at the middle belly of injured muscle was taken. Size of 150 representative regenerating myofibers with centrally located nuclei in injured muscle was blindly measured from 4 slides with 3 consecutive sections in each slide, using Axiovert software (Carl Zeiss Microscopy).

#### ***Single cell gel electrophoresis assay (Comet assay)***

3000 satellite cells were double-sorted into Eppendorf tubes containing 350µl of Hank's Buffered Salt Solution (Gibco) with 2% donor bovine calf serum, and comet assay was performed according to manufacturer's specifications (Cell Biolabs, Inc.). Cells were centrifuged at 700 rcf for 4 minutes at room temperature and the cell pellet was resuspended with low-melting point agarose gel pre-incubated at 37°C for at least 20 min. The cell-agarose suspension was then applied onto the comet slides gently and allowed to form a thin layer. The agarose layer was further solidified on ice before submersion in lysis buffer (Cell Biolabs, Inc.). Cell lysis was carried out 16-18 hours at 4°C and comet slide was then electrophoresed in alkaline electrophoresis buffer. Extreme care was taken to minimize exposure to light during sorting and subsequent processing and handling of cells until electrophoresis. Finally, electrophoresed DNA was stained with VistaDye and visualized using a Zeiss Imager M1 Fluorescence microscope (Carl Zeiss). Approximately 300 comets per animal were visually scored using a 3-point ranking scale and according to published protocols (Collins, 2004). This



scoring approach has been documented to be equally effective for detecting differences as other methods (e.g. calculation of tail moment) (Collins, 2004).

### ***Immunofluorescence***

For immunofluorescence, cells or frozen muscle sections were fixed in 4% paraformaldehyde in PBS for 20 minutes and washed with PBS. Cells or muscle sections were permeabilized and blocked using 2% BSA / 0.5% Goat Serum / 0.5% Triton-X in PBS for 60 minutes at room temperature. For primary antibodies raised in mouse, the M.O.M immunodetection kit (Vector Laboratories) was used. Primary antibodies (mouse monoclonal anti-fast myosin (1:200, Sigma), anti-myod (1:50, Santa Cruz) were incubated with samples for 12-16 hours at 4°C, and secondary antibodies (1:200, AlexaFluor 555, AlexaFluor 488 or AlexaFluor 594 Invitrogen) for 60 minutes at room temperature (with 3-4 washes in PBS following each incubation). All samples were mounted on slides in Vectashield mounting medium containing DAPI (Vector Laboratory). Fluorescence images were obtained using a Zeiss Imager M1 Fluorescence microscope (Carl Zeiss) and quantified using ImageJ.

### ***RNA transcriptome analysis***

Total RNA was extracted from muscle tissue using Trizol (Invitrogen). The quality of RNA was assessed with a 2100 Bioanalyzer (Agilent Technologies). RNA transcriptome analysis was performed using Gene chip WT PLUS reagent kit (Affymetrix) and Mouse Gene 2.0 ST arrays (Affymetrix). The dataset was normalized by RMA algorithm. A subset of differentially expressed myogenic transcripts was chosen to generate a heatmap from the original dataset normalized by gcRMA algorithm and SAM cutoff (<10% q-values) using R statistical software.

### ***In vivo electroporation***

TA muscles subjected to *in vivo* electroporation were pre-conditioned by injection of 10  $\mu$ l of 2 mg/ml hyaluronidase solution 1 hour prior to electroporation. Mixture of 50 pmol of siRNA (Life Technologies) and 30  $\mu$ g of mCherry-expressing plasmid (Addgene) in 10  $\mu$ l of PBS was injected into the pre-conditioned muscles and was allowed to wait 10 minutes before electroporation. Electrodes were protruded into each ventral end of TA muscle and electroporation was performed by applying 10 pulses, 50 ms in duration/each, at 1Hz. This procedure was repeated with negative control siRNA (Life Technologies) on the contralateral muscle.

### ***Satellite cell transplantation***

TA muscles of recipient mice were injured (25 $\mu$ l dose of 0.03mg/ml cardiotoxin) one day prior to injection in order to facilitate engraftment of fluorescent satellite cells. The next day, double-sorted satellite cells isolated from GFP transgenic mice, and recipient mice received 5000 double-sorted GFP<sup>+</sup> satellite cells per TA muscle. Four weeks post-transplant, TAs were harvested and promptly frozen in 2-methylbutane cooled in liquid nitrogen. Frozen samples were serially sectioned throughout the TA muscle from tendon to belly using Microm HM550 cryostat (Thermo Scientific). For analysis, number of GFP<sup>+</sup> fibers in each muscle section was counted to determine the maximum engraftment in each muscle. Muscle sections were analyzed blindly for maximum number of GFP<sup>+</sup> fiber and cross-sectional area of engrafted fibers using Axiovision software (Carl Zeiss).

### ***Western blot analysis***

Protein was extracted from whole muscle using Dounce homogenizers (Sigma) and COmplete Mini Protease Inhibitor Cocktail tablets (Roche) dissolved in M-PER protein extraction buffer (Pierce). Homogenates were centrifuged at 14,000g for 15min, after which the supernatant was removed for protein analysis. Protein concentrations of samples were measured with the BCA Protein Assay (Pierce), and samples were heated to 100°C for denaturing in Laemmli Sample Buffer (Bio-Rad) and  $\beta$ -mercaptoethanol for 10min.

Protein extracts were loaded on a 4–20% gradient Criterion Tris-HCl polyacrylamide gels (Bio-Rad) and then transferred to a PVDF membrane (Immuno-Blot, Bio-Rad). Membranes were blocked in 5% non-fat dry milk for 1h and were then stained at 4°C with primary antibody diluted 1:1000 in Tris-buffered saline (Atlanta Biologicals) with 0.1% Tween-20 and 5% milk. HRP-conjugated secondary antibodies (Santa Cruz) were diluted 1:2000 in 5% milk for 90min incubation at room temperature. Immunoblots were developed with an ECL plus system (Amersham) for visualization. A list of antibodies used is as follows: anti-GAPDH (ab9485, Abcam), anti-IL-6 (sc-1265, Santa Cruz), anti-HSP72 (adi-SPA-810-d, Enzo), anti-poly-Ubiquitin (Ab7254, Abcam), anti-Mouse IgG HRP Conjugated (sc-2005, Santa Cruz), Anti-Goat/Sheep HRP Conjugated (AB324P, Millipore).

### ***ELISA***

For ELISA, plasma harvested from young and aged mice were diluted as indicated and used on IL-6 ELISA kit (R&D System) as per manufacturer's instructions.

### ***Wire hang test***

In order to assess whole-body muscle strength and endurance, mice were allowed to grip a wire mesh grid with all four limbs. The grid was then inverted and placed at a height of 35

cm above a padded surface. Latency-to-fall was recorded in three successive timed trials. Mice were subjected to this test three separate times over the period of one week.

### ***Treadmill exercise***

In order for mice to become accustomed to the treadmill-exercise environment, conditioning was conducted for three days in a row for 10min/day at speeds increasing from 10m/min to 15.5m/min on a 15° upward incline. After this period, mice were allowed one day of rest. The following day, mice were run 15° uphill for one hour at 15.5m/min and then sacrificed immediately for whole muscle harvest.

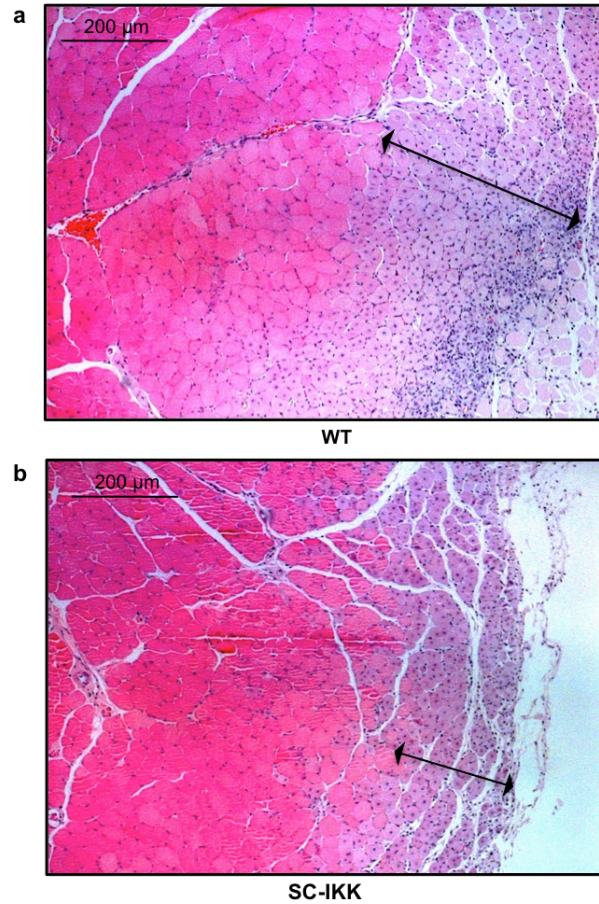
### ***Denervation***

Mice were anesthetized under isoflurane. Skin incision was made at the dorsal part of left thigh and sciatic nerve was exposed by blunt dissecting the posterior thigh muscles. Exposed nerve was cut and the skin incision was sutured. For sham injury control, the skin incision was sutured without cutting the sciatic nerve. Hind limb muscles were harvested 7, 14, 21 days after denervation and weighed to assess denervation induced muscle atrophy.

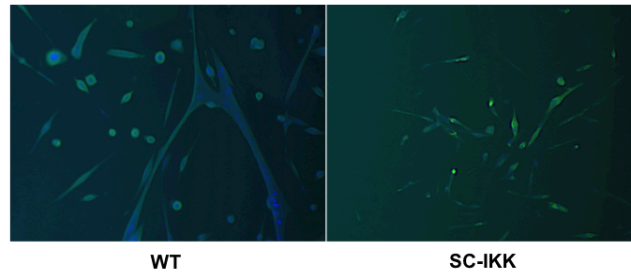
### ***Statistical analysis***

To determine statistical significance between experimental groups, I used the two-tailed, unpaired Student's *t*-test and one-way ANOVA (Microsoft Excel, Prism). Error bars in figures depict standard error of the mean (S.E.M.). Stepdown-Bonferroni method was used for analysis of difference between size distributions of two difference groups of mice, and signed rank test was used for comparing for non-parametric, paired data. Statistical significance was set at  $p < 0.05$ .

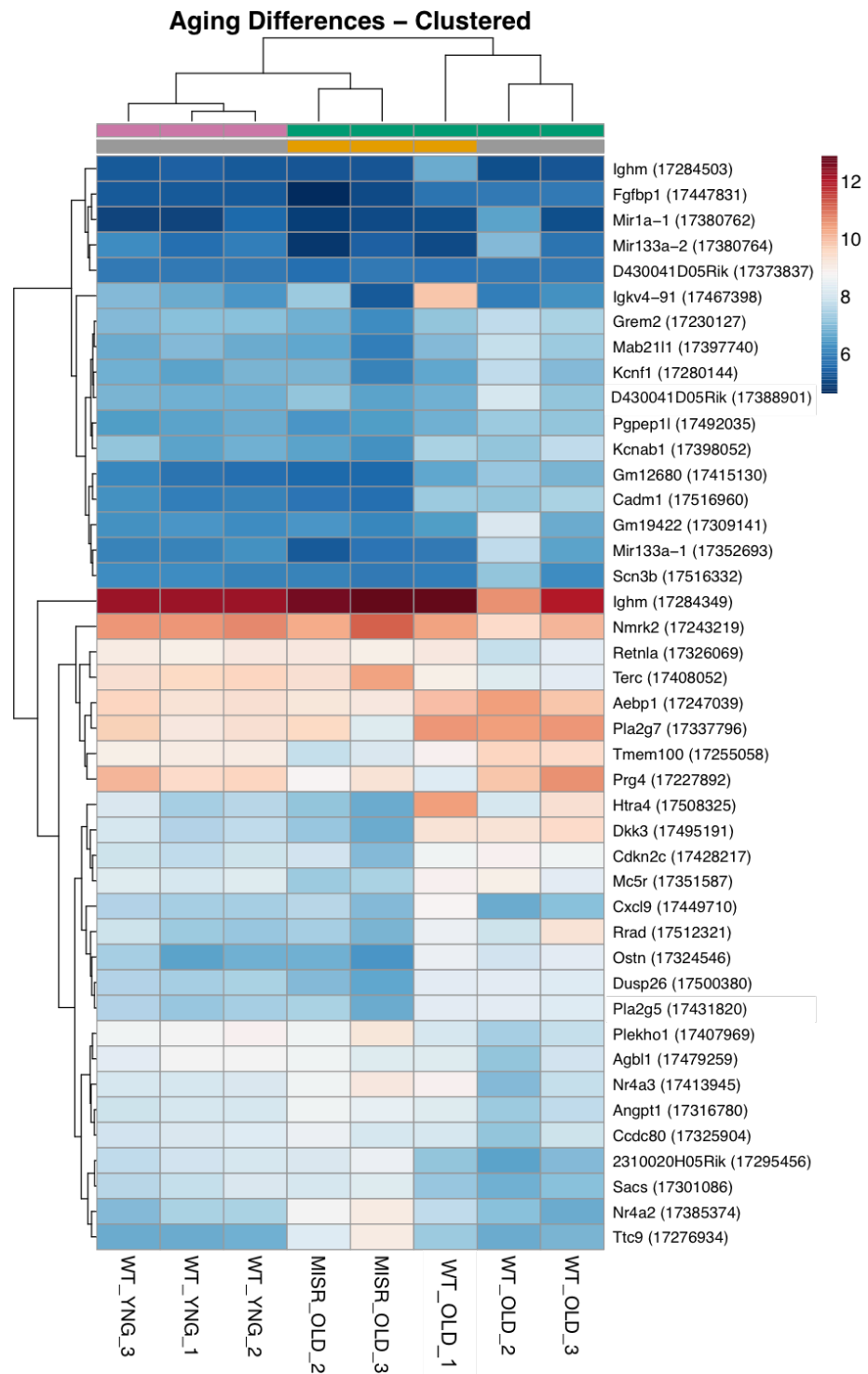
## **Appendix: Supplemental figures**



**Supplemental figure 1.** Reduced muscle regeneration in SC-IKK mice at 7 days after dry ice injury. Regenerating area of WT (a) and SC-IKK (b) muscle was compared by the perpendicular distance from the surface of cryoinjury to inner part of muscle where regenerating area ends.



**Supplemental figure 2.** Reduced myogenic differentiation of SC-IKK satellite cells in culture. Myotube formation (marked by myosin heavy chain staining) was reduced in satellite cells isolated from SC-IKK mice as compared to satellite cells of WT mice. (GFP: myosin heavy chain; DAPI: nucleus)



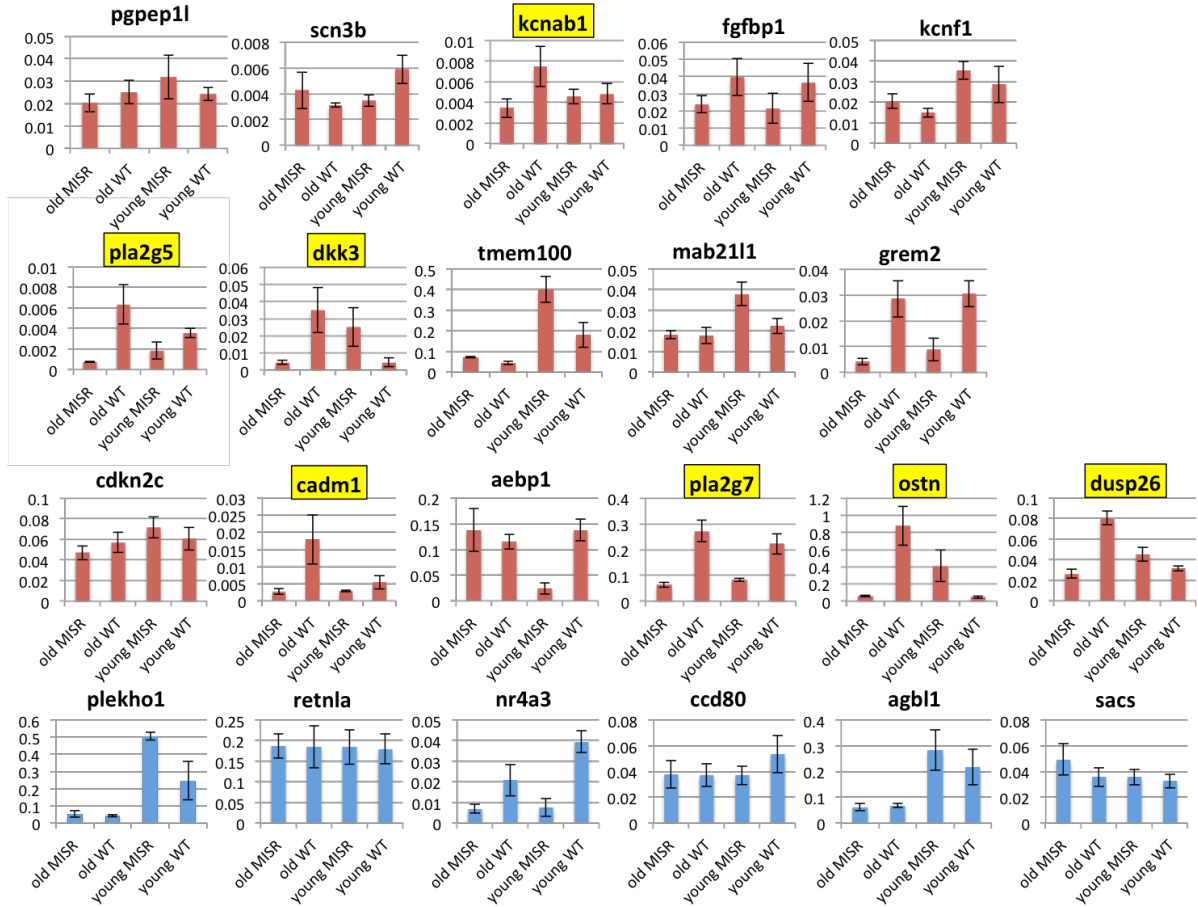
**Supplemental figure 3.** Heat map generated for candidate genes from gene array analysis of muscle tissue from young WT, aged WT, and aged MISR.



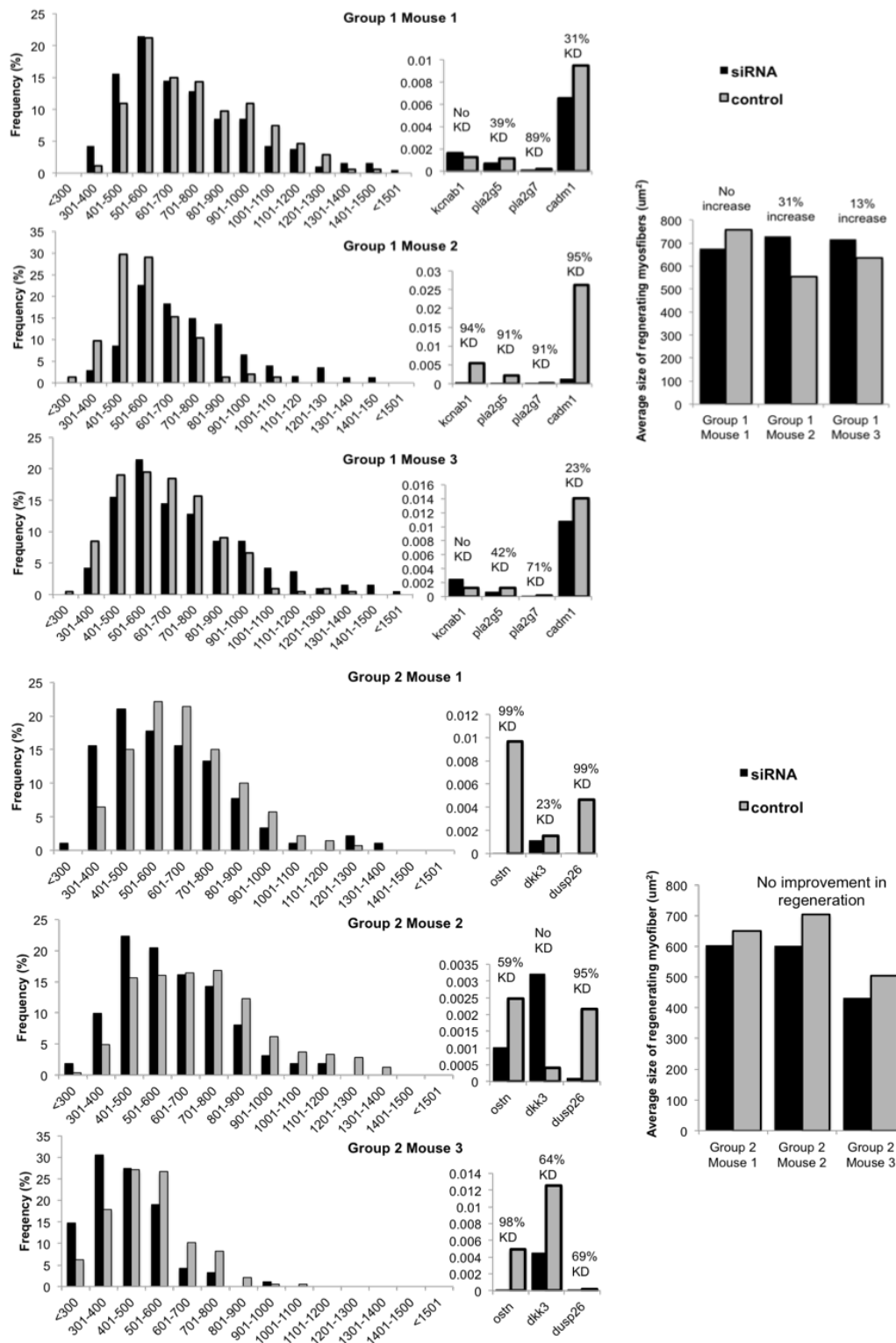
Genes up-regulated in age WT, down-regulated in young WT, and down-regulated in aged MISR		
	Function	NF-kB binding prediction
<i>Tmem100</i>	Trans-membrane protein	0.86
<i>Grem2</i>	Cytokine that inhibits the activity of BMP2 and BMP4 in a dose-dependent manner. Antagonized BMP4-induced suppression of progesterone production in granulose cells	N/A
<i>Pgpep1l</i>	Pyroglutamyl-Peptidase 1-Like Protein; cysteine-type peptidase activity	0.96
<i>Fgfbp1</i>	Fibroblast growth factor binding protein 1: interact with FGF-7, FGF-10, FGF-22	N/A
<i>Mab21l1</i>	Required for several aspects of embryonic development including normal development of the eye	N/A
<i>Kcnf1</i>	Voltage-gated potassium channel subunit	N/A
<i>Scn3b</i>	Sodium channel subunit beta3	0.93
<i>Aebp1</i>	Adipocyte enhancer binding protein1: May also positively regulate NF-kB activity in macrophages, leading to enhanced macrophage inflammatory responsiveness	N/A
<i>Kcnab1</i>	Potassium Voltage-Gated Channel, Shaker-Related Subfamily, Beta Member 1	0.91
<i>Mir1a-1</i>	Posttranslational regulation of gene expression by miRNA	0.89
<i>Pla2g5</i>	Phospholipase A2 group V. Secretion of this enzyme is thought to induce inflammatory responses in neighboring cells.	0.94
<i>Pla2g7</i>	Phospholipase A2 group VII. Catalyzes the degradation of platelet-activating factor to biologically inactive products.	0.81
<i>Dkk3</i>	Glycoproteins that antagonizes canonical Wnt signaling	0.84
<i>Cdkn2c</i>	P18-INK4C; G1 to S cell cycle reactome	N/A
<i>Cadm1</i>	Cell adhesion molecule 1: mediates homophilic cell-cell adhesion in a Ca(2+)-independent manner;.	0.87
<i>Ostn</i>	Musclin: muscle-derived secreted factor. Appears to modulate osteoblastic differentiation.	N/A
<i>Dusp26</i>	Dual specificity phosphatase 26: Inactivates MAPK1 and MAPK3 which leads to dephosphorylation of heat shock factor protein4 and a reduction in its DNA-binding activity. Inhibits MAP kinase p38	N/A

Genes down-regulated in age WT, up-regulated in young WT, and up-regulated in aged MISR		
	Function	NF-kB binding prediction
<i>Ccdc80</i>	Promotes cell adhesion and matrix assembly	0.92
<i>Agbl1</i>	ATP/GTP protein binding protein-like 1: metallopeptidase activity and tubulin binding	N/A
<i>Nmrk2</i>	Nicotinamide riboside kinase 2	0.99
<i>Sacs</i>	Co-chaperone which acts as a regulator of the Hsp70 chaperone machinery and may be involved in the processing of other ataxia-linked proteins; interact with NF-kB	0.86
<i>Plekho1</i>	Pleckstrin homology domain containing family O member 1: regulation of the actin cytoskeleton through its interactions with actin capping protein.	N/A
<i>Retnla</i>	Resistin like alpha: secreted protein, expressed in adipose tissue	0.83
<i>Terc</i>	Telomerase RNA component	0.98
<i>Nr4a3</i>	A member of the steroid-thyroid hormone-retinoid receptor superfamily: nuclear receptor subfamily4 groupA	0.86

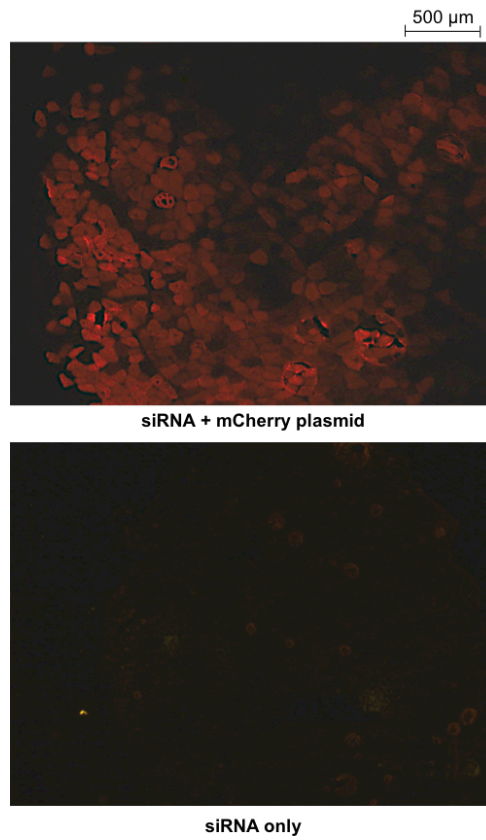
**Supplemental table 1.** Functions and NF-kB binding prediction of candidate genes selected from gene array analysis in muscle tissue of young and aged WT and aged MISR.



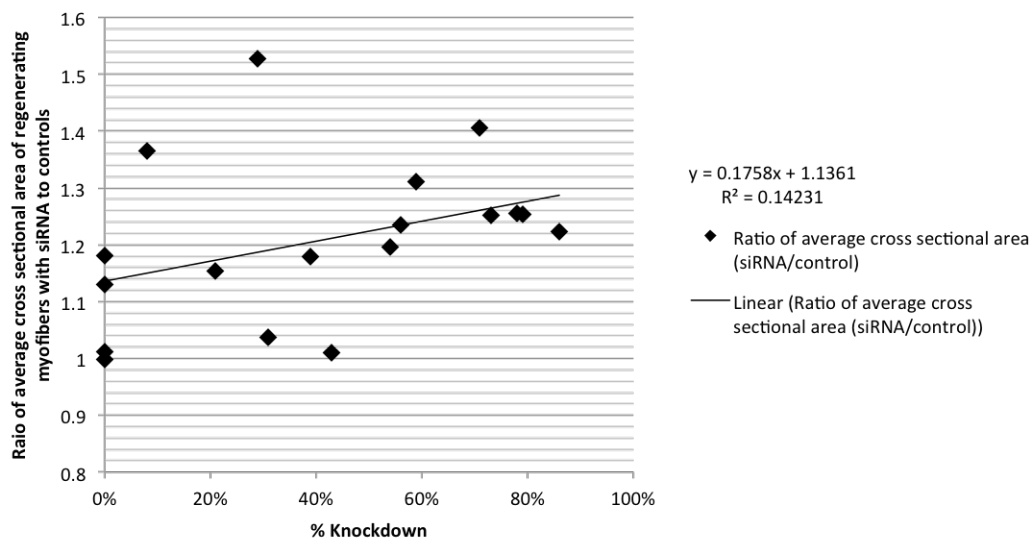
**Supplemental figure 4.** qRT-PCR analysis on candidate gene expression in young and old WT and MISR muscle for validation of gene array results. (Validated genes are highlighted)



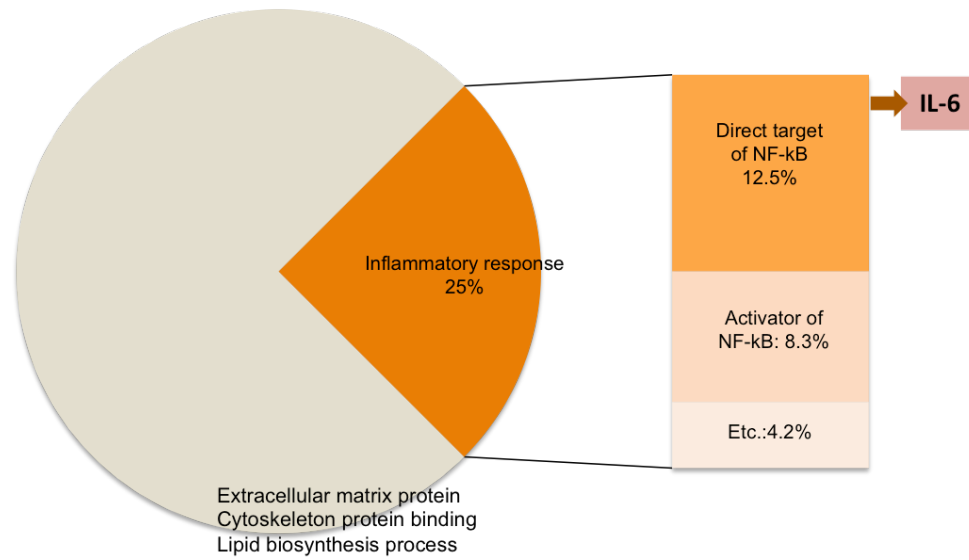
**Supplemental figure 5.** Results from pooled screen analysis of candidate genes. siRNAs for each group of candidate genes were in vivo electroporated into TA muscles of aged mice in order to screen for genes that has impact on muscle regeneration.



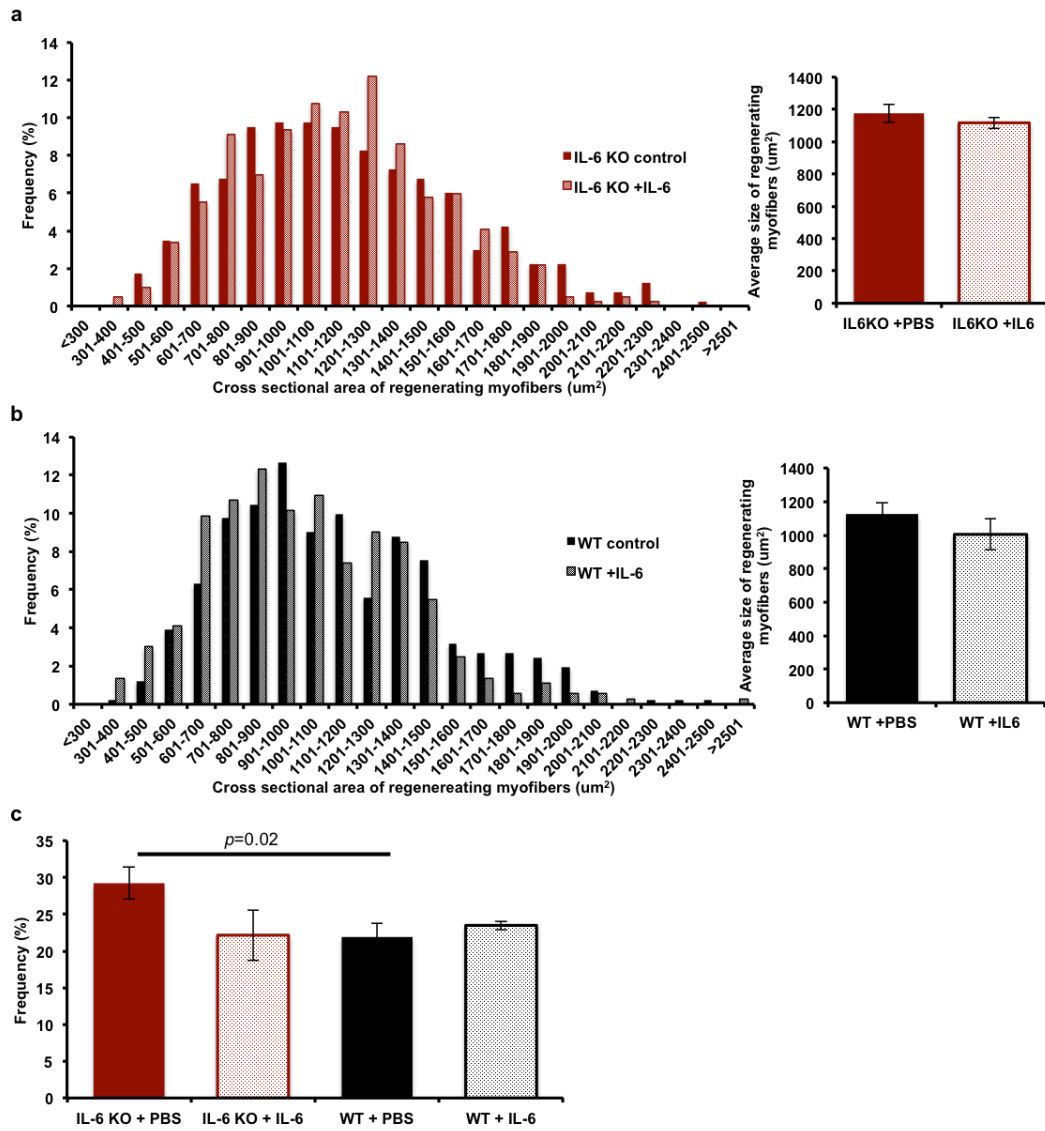
**Supplemental figure 6.** *In vivo* electroporation and expression of mCherry fluorescent protein-expressing plasmid. mCherry fluorescent protein-expressing plasmid (30ug) was co-electroporated with siRNA and its expression was checked as a reporter of electroporation efficiency.



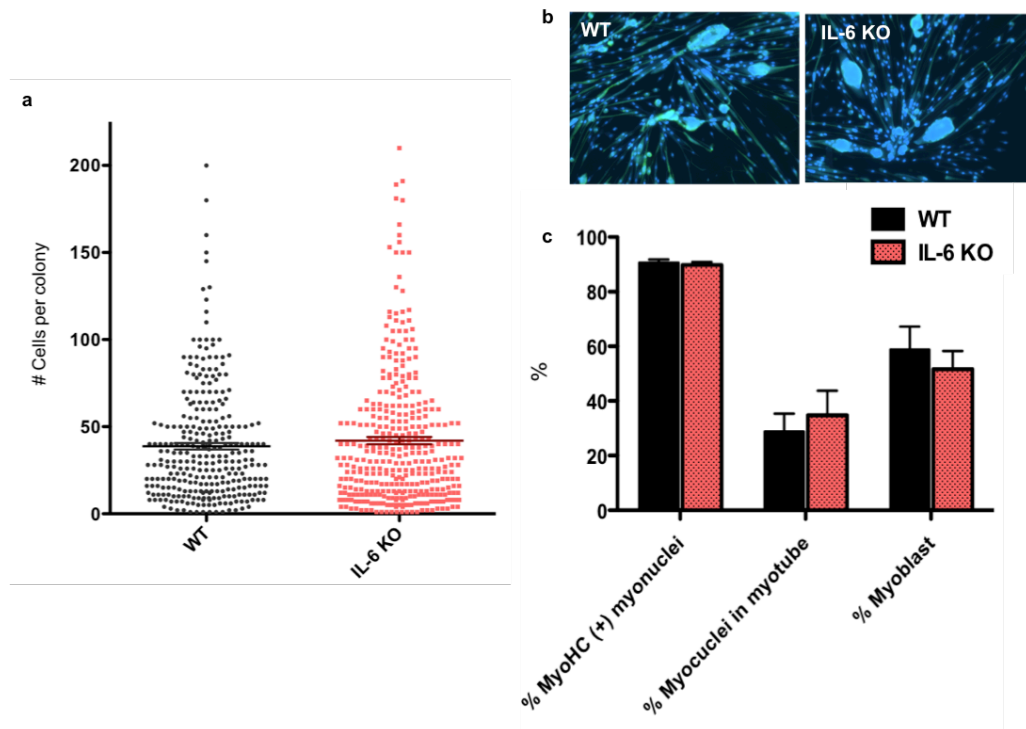
**Supplemental figure 7.** Linear regression analysis on the correlation between the level of pla2g5 gene knockdown and the level of improvement in muscle regeneration in aged mice.



**Supplemental figure 8.** Composition of top 10% genes highly up-regulated in aged satellite cells assessed by gene array analysis of young and aged satellite cells.

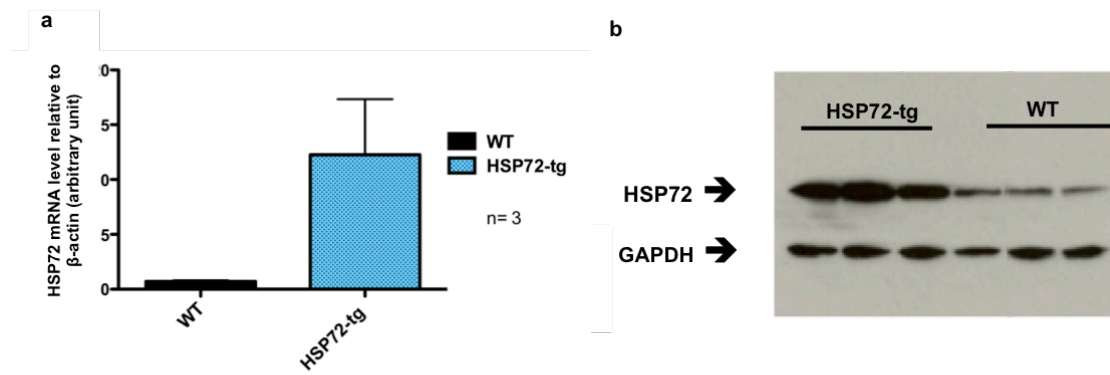


**Supplemental figure 9.** Prolonged IL-6 exposure in young IL-6 KO and WT mice. Daily injection of IL-6 for chronic exposure to IL-6 in circulation did not result in changes in muscle regeneration either in IL-6 KO (a) or WT mice (b). (c) Colony formation efficiency of satellite cells from IL-6 KO mice and WT mice injected daily with recombinant IL-6 or vehicle (PBS). IL-6 injection does not affected myogenic colony formation both in IL-6 KO and WT mice. All data are presented as mean  $\pm$  SEM.



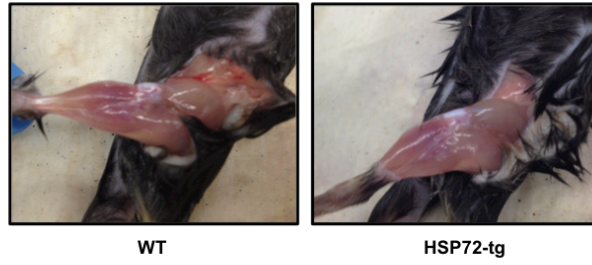
**Supplemental figure 10.** IL-6 KO satellite cells does not show difference in proliferation or differentiation. (a) Scatter plot comparing the size of myogenic colony after 5 days of satellite cell culture. Number of cells per colony was counted as a measurement of colony size. (b) Satellite cells of IL-6 KO and WT mice were cultured and induced to differentiate and form myotubes. Myotubes were immunostained by myosin heavy chain (GFP) and myonucleus was stained by DAPI. (c) Satellite cells of IL-6 KO mice and WT mice did not show difference in the differentiation potential measured by the number of myosin heavy chain<sup>+</sup> myonuclei, number of myonuclei in myotubes, and number of myonuclei in elongated cells out of total number of nuclei in each well. All data are presented as mean  $\pm$  SEM.



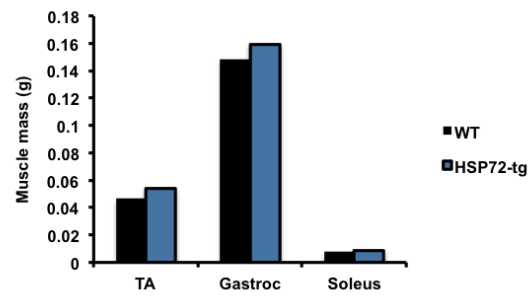


**Supplemental figure 11.** Validation of HSP72 overexpression in transgenic mice by pRT-PCR for transcription level (a) and western blot analysis for protein level (b). All data are presented as mean  $\pm$  SEM.

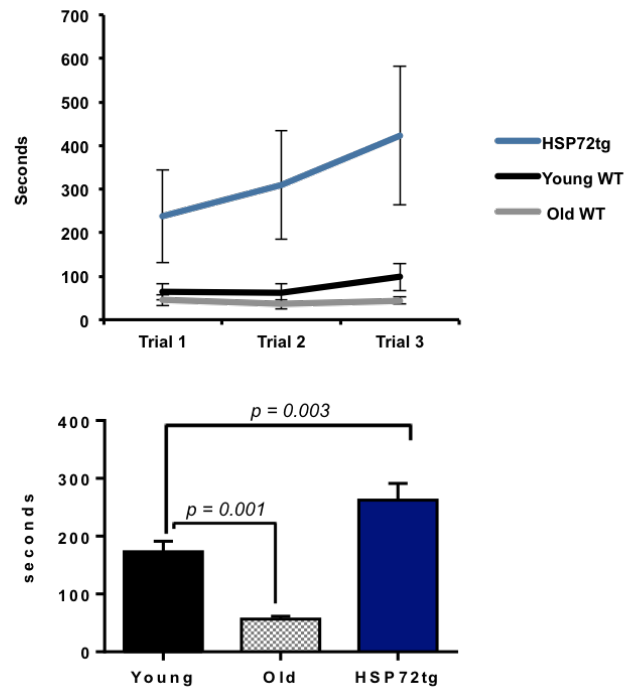
a



b



**Supplemental figure 12.** Muscle phenotype of HSP72-tg mice is not different from WT. (a) Size of hindlimb muscles are not visibly different between HSP72-tg mice and WT mice. (b) Quantification of hindlimb muscle mass of HSP72-tg and WT mice.



**Supplemental figure 13.** HSP72-tg mice show increased muscle strength and endurance. Wire hang test performed on young HSP72-tg mice and young and old WT mice. HSP72-tg mice showed significantly improved latency to fall. All data are presented as mean  $\pm$  SEM.

Gene	Forward primer	Reverse primer
<i>Actb</i>	5'-TTCTGGTGCTTGTCTCACTGA-3'	5'-CAGTATGTTCTGGCTTCCCATTG-3'
<i>Il6</i>	5'-GTTCTCTGGGAAATCGTGGA-3'	5'-TCTGCAAGTTGCATCATCGTT-3'
<i>Il33</i>	5'-TGCCTCTGTTGACACATTGA-3'	5'-STGTACTCAGGGAGGCAGGA-3'
<i>Cox2</i>	5'-TGAGCAACTATTCCAAACCAGC-3'	5'-GCACGTAGTCTTCGATCACTATC-3'
<i>Ccl2</i>	5'-AAGAGGATCACCAGCAGCAG-3'	5'-TCTGGACCCATTCTTCTTG-3'
<i>IL-6</i>	5'-GTTCTCTGGGAAATCGTGGA-3'	5'-TCTGCAAGTGCATCATCCGTT-3'
<i>HSP72</i>	5'-ACCAAGCAGACGCAGATCTTC-3'	5'-GCCCTCGTACACCTGGATCA-3'
<i>Myogenin</i>	5'-CTACAGGCCTTGCTCAGCTC-3'	5'-GTGGGAGTTGCATTCACTGG-3'
<i>pla2g5</i>	5'-CCAGGGGGCTTGCTAGAAC-3'	5'-AGCACCAATCAGTGCCATCC-3'
<i>Plekho1</i>	5'-AATTCTGCGGGAAAGGGATT-3'	5'-AACACCTCCTGACTGTTTTCTC-3'
<i>Retnla</i>	5'-CCAATCCAGCTAACTATCCCTCC-3'	5'-ACCCAGTAGCAGTCATCCCA-3'
<i>Nr4a3</i>	5'-AGGATTCACTGATCTCCCCAA-3'	5'-GATGCAGGACAAGTCCATTGC-3'
<i>Ccd80</i>	5'-CCTGCCTTGATAGCGACAG-3'	5'-ACACTGGTACACTCTTCCTCC-3'
<i>Abg1l</i>	5'-CCAGCAGTGCCTATACCTTCC-3'	5'-TGCTCAGATCAGTTTCCAAGTC-3'
<i>Sacs</i>	5'-TCAGTTTGCCCCCTTCATTGG-3'	5'-CTGTAAGCGAAGAGGGAAACG-3'
<i>Dusp26</i>	5'-ATGCCCTCTGTTACCATCC-3'	5'-CTGTTGTGTGAGGCGTTGAG-3'
<i>Ostrn</i>	5'-CGTCTTGATGATCTGGTGTCC-3'	5'-TGGGAATACCAAACCGCTTTT-3'
<i>Pla2g7</i>	5'-CTTTTCACTGGCAAGACACATCT-3'	5'-CGACGGGGTACGATCCATTTC-3'
<i>Aebp1</i>	5'-TTGGAAACGCTGGATCGGTTA-3'	5'-CTTGACCTTGCCAGGCATTT-3'
<i>Cadm1</i>	5'-GAACCAGCAGTTCACGATTCT-3'	5'-AGCAAGCATAGCATGGCAAAC-3'
<i>Cdkn2c</i>	5'-CCTTGGGGGAACGAGTTGG-3'	5'-AAATTGGGATTAGCACCTCTGAG-3'
<i>Dkk3</i>	5'-CTCGGGGGTATTTGCTGTGT-3'	5'-TCCTCCTGAGGGTAGTTGAGA-3'
<i>Trem100</i>	5'-GACAATGGAGAAAAACCCCAAGA-3'	5'-GGTAGCAGGAGAGTTCGGC-3'
<i>Mab21l1</i>	5'-CCAAGCTGGTCTACACCTG-3'	5'-GCGGTTCTGCACTTCAAC-3'
<i>Grem2</i>	5'-GGTAGCTGAAACACGGAAGAA-3'	5'-TCTTGCAACAGTCACTCTTGA-3'
<i>Kcnf1</i>	5'-CGTGGCAGGCGAAGACATT-3'	5'-CCCCCGCCAAACAGTTGAT-3'
<i>Fgfbp1</i>	5'-GGCAACTCAGGCGTTCTCA-3'	5'-CGTCAGAGATTTAGATGTCCTGC-3'
<i>Kcnab1</i>	5'-AGGACCGACTTCTGAGCAAG-3'	5'-GATAGCGACAGTGCAGGAATTT-3'
<i>Scn3b</i>	5'-GATTGCTTCCCCTAGCTTCTCT-3'	5'-AGGAAATCTTTACCGCCCTCA-3'
<i>Pgpep1l</i>	5'-AGGCCATCTTCTGGAACAGT-3'	5'-GCCACTTCGACATTCTCAACAG-3'

**Supplemental table 2.** List sequences of all primers utilized in qRT-PCR.

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